

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 August 2001 (16.08.2001)

PCT

(10) International Publication Number
WO 01/58493 A1

(51) International Patent Classification⁷: **A61K 47/48**,
38/24, C07K 14/59

(21) International Application Number: PCT/DK01/00090

(22) International Filing Date: 9 February 2001 (09.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2000 00220 11 February 2000 (11.02.2000) DK
PA 2000 01092 14 July 2000 (14.07.2000) DK

(71) Applicant: **MAXYGEN APS** [DK/DK]; Agern Allé 1,
DK-2970 Hørsholm (DK).

(72) Inventors: **SCHAMBYE, Hans, Thalsgård**; Maxygen
ApS, Agern Allé 1, DK-2970 Hørsholm (DK). **AN-
DERSEN, Kim, Vilbourn**; Maxygen ApS, Agern Allé 1,
DK-2970 Hørsholm (DK). **VAN DEN HAZEL, Bart**;
Maxygen ApS, Agern Allé 1, DK-2970 Hørsholm (DK).
CHRISTIANSEN, Jesper; Maxygen ApS, Agern Allé 1,
DK-2970 Hørsholm (DK). **JEPPESEN, Claus, Bekker**;
Maxygen ApS, Agern Allé 1, DK-2970 Hørsholm (DK).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: FOLLICLE STIMULATING HORMONES

(57) Abstract: The invention relates to heterodimeric polypeptide conjugates exhibiting FSH activity, comprising a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein at least one of the FSH- α and FSH- β subunits differs from the corresponding wildtype subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed, and having at least one non-polypeptide moiety bound to an attachment group of at least one of said subunits. Preferably, at least one attachment group, e.g. an N- or O-glycosylation site or an attachment site for a polymer molecule such as polyethylene glycol, has been introduced, e.g. at an N-terminal. The polypeptide conjugates exhibit improved properties, in particular an increased half-life, compared to human FSH.



WO 01/58493 A1

FOLLICLE STIMULATING HORMONES

Field of the invention

The present invention relates to new polypeptides and polypeptide conjugates exhibiting follicle stimulating hormone (FSH) activity, to methods for preparing such polypeptides and conjugates, and to the use of such polypeptides and conjugates in therapy, in particular in the treatment of infertility.

Background of the invention

Follicle Stimulating Hormone (FSH) is a dimeric hormone consisting of an α subunit and a β subunit. The α subunit is common to the glycoprotein hormone family, which apart from FSH includes chorionic gonadotropin (CG), thyroid stimulating hormone (TSH), and luteinizing hormone (LH), whereas the β subunit is specific to FSH. The human wildtype α subunit is a 92 amino acid glycoprotein, the amino acid sequence of which is shown in SEQ ID NO:2. Said subunit is referred to herein as hFSH- α . The human wildtype β subunit is a 111 amino acid glycoprotein that has the amino acid shown in SEQ ID NO:4. This subunit is referred to herein as hFSH- β .

Human FSH (hFSH) has been isolated from pituitary glands and from post-menopausal urine (EP 322 438) and has been produced recombinantly in mammalian cells (US 5,639,640, US 5,156,957, US 4,923,805, US 4,840,896, US 5,767,251, EP 211,894 and EP 521,586). The latter references also disclose the hFSH- β gene. US 5,405,945 discloses a modified human α subunit gene comprising only one intron.

US 4,589,402 and US 4,845,077 disclose purified hFSH which is free of LH and the use thereof for *in vitro* fertilization. EP 322 438 discloses a protein with at least 6200 U/mg FSH activity which is substantially free of LH activity, and wherein the FSH α subunit and β subunit, respectively, may be wildtype or specified truncated forms thereof.

Liu et al., *J Biol Chem* 1993, 15;268(2):21613-7, Grossmann et al., *Mol Endocrinol* 1996 10(6): 769-79, Roth and Dias (*Mol Cell Endocrinol* 1995 1; 109(2): 143-9, Valove et al., *Endocrinology* 1994; 135(6):2657-61, Yoo et al., *J Biol Chem* 1993 25; 268(18): 13034-42), US 5,508,261 and Chappel et al., 1998, *Human Reproduction*, 13(3): 18-35 disclose various structure-function relationship studies and identify amino acid residues involved in receptor binding and activation and in dimerization of FSH.

It has been found that glycosylation of FSH- α and FSH- β is essential for receptor signal transduction. hFSH- α comprises two N-glycosylation sites at the asparagines located at position 52 and 78, whereas hFSH- β comprises two N-glycosylation sites at the asparagines located at positions 7 and 24. The importance of the various N-glycosylation sites for the binding and signal-transducing activities of FSH are discussed, *inter alia*, by Valove et al., *Endocrinology* 1994; 135(6):2657-61 and Flack et al., *J Biol Chem* 1994 13;269(19):14015-20.

Galway et al., *Endocrinology* 1990; 127(1):93-100 demonstrate that FSH variants produced in a N-acetylglucosamine transferase-I CHO cell line or a CHO cell line defective in sialic acid transport are as active as FSH secreted by wildtype cells or purified pituitary FSH *in vitro*, but lacked *in vivo* activity, presumably due to rapid clearance of the inadequately glycosylated variants in serum. D'Antonio et al., *Human Reprod* 1999; 14(5):1160-7 describe various FSH isoforms circulating in the blood stream. The isoforms have identical amino acid sequences, but differ in their extent of post-translational modification. It was found that the less acidic isoform group had a faster *in vivo* clearance as compared with the acidic isoform group, possibly due to differences in the sialic acid content between the isoforms.

US 5,087,615 discloses a method for stimulating follicle development and ovulation in a female patient by administering FSH to said patient during the follicular phase of the ovulatory cycle, the improvement comprising initially administering a first FSH isoform having a relatively long plasma half-life and subsequently administering a second FSH isoform having a shorter plasma half-life.

Bishop et al. *Endocrinology* 1995; 136(6):2635-40 conclude that circulatory half-life appears to be the primary determinant of *in vivo* activity.

Attempts have been made to prolong the serum half-life of FSH. US 5,338,835 and US 5,585,345 disclose a modified FSH- β subunit extended at the C-terminal Glu with the carboxy terminal portion (CTP) region of hCG (the region consisting of the amino acid sequence which occurs from positions 112-118 to 145, and comprising four O-linked glycosylation sites located at positions 121, 127, 132 and 138). The resulting modified subunit is stated to have the biological activity of native FSH, but a prolonged circulating half-life. US 5,405,945 discloses that the carboxy terminal portion of the CG β subunit or a variant thereof has significant effects on the clearance of CG, FSH, and LH.

US 5,883,073 discloses single-chain proteins comprised of two α -subunits with agonist or antagonist activity for CG, TSH, LH and FSH.

US 5,508,261 discloses heterodimeric polypeptides having binding affinity to LH and FSH receptors comprising a glycoprotein hormone α subunit and a non-naturally occurring β subunit polypeptide, wherein the β subunit polypeptide is a chain of amino acids comprising four joined subsequences, each of which is selected from a list of specific sequences.

US 5,567,422 and WO 98/32466 mention FSH among a vast number of other therapeutic proteins that may be PEGylated.

Currently, FSH is used therapeutically to stimulate the growth and maturation of ovarian follicles in infertile women. In particular, FSH is used in connection with *in vitro* fertilization as well as for the treatment of anovulatory women, with anovulatory syndrome or luteal phase deficiency. However, one problem encountered in current FSH treatment is the fairly short *in vivo* half-life of FSH requiring frequent, usually daily administration of the product. The frequent administration is very inconvenient for the patient and results in high fluctuations of FSH activity in the blood stream, which may cause inadequate maturation of the follicles.

Therefore, a clinical need exists for a product which provides part or all of the therapeutically relevant effects of FSH, and which may be administered at less frequent intervals as compared to currently available FSH product, and which preferably provides a more stable level of circulating FSH activity as compared to that obtainable by current treatment. The present invention is directed to such products as well as the means of making such products.

Brief disclosure of the invention

More specifically, the present invention relates to polypeptide conjugates exhibiting FSH activity and methods for their preparation and their use in medical treatment.

Accordingly, in its first aspect the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising i) a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein at least one of said FSH- α and FSH- β subunits differs from the corresponding wildtype subunit in that at least one amino acid residue acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or

removed, and ii) at least one non-polypeptide moiety bound to an attachment group of at least one of said subunits.

In another aspect the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising i) a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein the amino acid sequence of at least one of said FSH- α and FSH- β subunits differs from that of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced, and ii) at least one oligosaccharide moiety bound to an N-glycosylation site of at least one of said subunits.

In a further aspect, the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising FSH- α and FSH- β subunits, wherein at least one of said FSH- α and FSH- β subunits comprises, relative to the corresponding wildtype subunit, at least one introduced N- or O-glycosylation site at the N-terminal thereof, said at least one introduced glycosylation site being glycosylated.

In the above aspects the corresponding wildtype subunits are preferably hFSH- α and hFSH- β , respectively.

Another aspect of the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein at least one of said FSH- α and FSH- β subunits comprises a polymer molecule bound to the N-terminal thereof.

In a further aspect the invention relates to modified FSH- α and modified FSH- β polypeptides that may be used as intermediate products for the preparation of a conjugate with a polymer molecule.

In still further aspects the invention relates to methods for preparing a conjugate or a polypeptide of the invention, including nucleotide sequences and expression vectors encoding a polypeptide or a conjugate of the invention.

In final aspects the invention relates to a composition comprising a conjugate or polypeptide of the invention and methods of treating a mammal with such composition. In particular, the polypeptide, conjugate or composition of the invention may be used to treat infertility.

Description of the drawing and sequence listing

Figure 1 shows a sequence alignment of human FSH to the structural part of two published structures of human chorionic gonadotropin.

SEQ ID NO:1 is the complete amino acid sequence of the common α chain, the “glycoprotein hormones α chain” (Fiddes et al., *Nature* 281:351-356 (1979)). Rathnam et al., *J. Biol. Chem.* 250:6735-6746 (1975) reports residue Q29 to be a Glu. Sairam et al., *Can. J. Biochem.* 55:755-760 (1977), and Sairam et al., *Biochem. Biophys. Res. Commun.* 48:530-537 (1972) report the sequence CS at positions 108-109 to be SC. FSH- α variants having these changes are intended to be encompassed by the term “FSH- α ” as used herein.

SEQ ID NO:2 is the mature amino acid sequence of the common α chain shown in SEQ ID NO:1.

SEQ ID NO:3 is the complete amino acid sequence of the human FSH β chain (Tanzi et al., *DNA* 6:205-212(1987)).

SEQ ID NO:4 is the mature amino acid sequence of the human FSH β chain shown in SEQ ID NO:3.

SEQ ID NO:5, **SEQ ID NO:6** and **SEQ ID NO:7** are DNA sequences of plasmids described in the Examples.

Detailed disclosure of the invention**Definitions**

In the context of the present application and invention the following definitions apply:

The term “conjugate” is intended to indicate a heterogeneous molecule formed by the covalent attachment of one or more polypeptides to one or more non-polypeptide moieties such as polymer molecules, oligosaccharide moieties, lipophilic compounds, carbohydrate moieties or organic derivatizing agents. The term covalent attachment means that the polypeptide and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. The term “non-conjugated polypeptide” may be used about the polypeptide part of the conjugate.

The term "polypeptide" may be used interchangeably herein with the term "protein". Further, the terms "polypeptide" and "protein" are generally used herein for the sake of simplicity to refer to the heterodimeric FSH polypeptides/proteins and conjugates of the invention, even though these proteins strictly speaking comprise a dimer of the α and β polypeptide subunits. The individual subunits are referred to herein as FSH- α and FSH- β , respectively, so that it is clear from the context whether reference is made to the dimeric hormone or to one of the subunits.

The "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is intended to cover carbohydrate molecules attached by *in vitro* glycosylation. Carbohydrate molecules attached by *in vivo* glycosylation, such as N- or O-glycosylation (as further described below) are referred to herein as "an oligosaccharide moiety". Except where the number of polymer molecules is expressly indicated, every reference to "a polymer", "a polymer molecule", "the polymer" or "the polymer molecule" contained in polypeptide of the invention or otherwise used in the present invention shall be a reference to one or more polymer molecule(s).

The term "attachment group" is intended to indicate an amino acid residue group of the polypeptide capable of coupling to the relevant non-polypeptide moiety. For instance, for polymer conjugation to PEG, a frequently used attachment group is the ϵ -amino group of lysine or the N-terminal amino group. Other polymer attachment groups include a free carboxylic acid group (e.g. that of the C-terminal amino acid residue or of an aspartic acid or glutamic acid residue), suitably activated carbonyl groups, oxidized carbohydrate moieties and mercapto groups. Useful attachment groups and their matching non-peptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-peptide moiety	Conjugation method/- Activated PEG	Reference
-NH ₂	N-terminal, Lys, His, Arg	Polymer, e.g. PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al., critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)

-COOH	C-term, Asp, Glu	Polymer, e.g. PEG, with ester or amide group Oligosaccharide moiety	mPEG-Hz <i>In vitro</i> coupling	Shearwater Inc.
-SH	Cys	Polymer, e.g. PEG, with disulfide, maleimide or vinyl sulfone group Oligosaccharide moiety	PEG-vinylsulphone PEG-maleimide <i>In vitro</i> coupling	Shearwater Inc. Delgado et al., critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-OH	Ser, Thr, -OH, Lys	Oligosaccharide moiety PEG with ester, ether, carbamate, carbonate	<i>In vivo</i> O-linked glycosylation	
-CONH ₂	Asn as part of an N-glycosylation site	Oligosaccharide moiety Polymer, e.g. PEG	<i>In vivo</i> N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Oligosaccharide moiety	<i>In vitro</i> coupling	
-CONH ₂	Gln	Oligosaccharide moiety	<i>In vitro</i> coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759-65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g. PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179:301, WO 92/16555, WO 00/23114
Guanidino	Arg	Oligosaccharide moiety	<i>In vitro</i> coupling	Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Oligosaccharide moiety	<i>In vitro</i> coupling	As for guanidine

For *in vivo* N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting an N-glycosylation site (with the sequence N-X'-S/T/C-X'', wherein X' is any amino acid residue except proline, X'' any amino acid residue which may or may not be identical to X' and which preferably is different from proline, N is asparagine, and S/T/C is either serine, threonine or cysteine, preferably

serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is where the oligosaccharide moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site are present. Accordingly, when the non-peptide moiety is an oligosaccharide moiety and the conjugation is to be achieved by N-glycosylation, the term “amino acid residue comprising an attachment group for the non-peptide moiety” as used in connection with alterations of the amino acid sequence of the polypeptide of interest is to be understood as meaning that one or more amino acid residues constituting an N-glycosylation site are to be altered in such a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

In the present application, amino acid names and atom names (e.g. CA, CB, NZ, N, O, C, etc.) are used as defined by the Protein DataBank (PDB) (www.pdb.org), which is based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names etc.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985). The term “amino acid residue” is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e. alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions/substitutions is illustrated as follows: E9(a) indicates position number 9 occupied by a glutamic acid residue in the amino acid sequence shown in SEQ ID NO:2. E9(a)N indicates that said glutamic acid residue has been substituted by an asparagine residue. Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence shown in SEQ ID NO:2 (for FSH- α , indicated by “(a)”) or SEQ ID NO:4 (for FSH- β , indicated by “(b)”). Multiple substitutions are indicated with a “+”, e.g. M109(b)N+E111(b)S/T means an amino acid sequence which comprises substitution of the methionine residue in position 109 of FSH- β by an asparagine residue and substitution of the glutamic acid residue in position 111 in FSH- β by a serine or a threonine residue.

The term “nucleotide sequence” is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combination thereof.

The term “polymerase chain reaction” or “PCR” generally refers to a method for amplification of a desired nucleotide sequence *in vitro*, as described, for example, in US 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

“Cell”, “host cell”, “cell line” and “cell culture” are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell. “Transformation” and “transfection” are used interchangeably to refer to the process of introducing DNA into a cell.

“Operably linked” refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The term “introduce” refers to introduction of an amino acid residue comprising an attachment group for a non-polypeptide moiety, either by substitution of an existing amino acid residue or by insertion of an additional amino acid residue. The term “remove” refers to removal of an amino acid residue comprising an attachment group for a non-polypeptide moiety, either by substitution of the amino acid residue to be removed by another amino acid residue or by deletion (without substitution) of the amino acid residue to be removed.

When substitutions are performed in relation to a parent polypeptide, they are preferably “conservative substitutions”, in other words substitutions performed within groups

of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

Preferred substitutions in the present invention may in particular be chosen from among the conservative substitution groups listed in the table below.

5

Conservative substitution groups:

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system.

10 The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally reduced antibody reactivity will be an indication of a reduced immunogenicity. The reduced immunogenicity may be determined by use of any suitable method known in the art, e.g. *in vivo* or *in vitro*.

15 The term "functional *in vivo* half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide or conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the initial value. As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, i.e. the time at which 50% of the dispensed polypeptide or conjugate
20 molecules is still present in the circulation/plasma/bloodstream. The magnitude of serum half-life is usually a good indication of the magnitude of functional *in vivo* half-life. Alternative terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide or conjugate is cleared by the action of one or more of the kidney, reticuloendothelial systems (RES), spleen or liver, by
25 FSH-receptor-mediated elimination, or by specific or non-specific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functional *in*

in vivo half-life and the serum half-life may be determined by any suitable method known in the art as further discussed in the Examples section hereinafter.

The term "increased" as used about the functional *in vivo* half-life or serum half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is statistically significantly increased relative to that of a reference molecule, such as a non-conjugated rhFSH (recombinant hFSH), e.g. Gonal-F® (available from Serono) or Puregon® (available from Organon), as determined under comparable conditions. For instance, the relevant half-life may be increased by at least about 25%, such as by at least about 50%, e.g. by at least about 100%, 200% or 500%.

The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or tubular elimination. Renal clearance depends on physical characteristics of the conjugate, including size (diameter), symmetry, shape/rigidity and charge. Reduced renal clearance may be established by any suitable assay, e.g. an established *in vivo* assay. Typically, renal clearance is determined by administering a labelled (e.g. radioactive or fluorescent labelled) polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g. the corresponding non-conjugated polypeptide, a non-conjugated corresponding wild-type polypeptide or another conjugated polypeptide (such as a conjugated polypeptide not according to the invention), under comparable conditions.

In some cases, it will be preferred to obtain a clearance that is only slightly reduced (i.e. total clearance by renal clearance, receptor-mediated clearance and/or other clearance mechanisms), e.g. to increase the *in vivo* half-life from about 24 hours to about 3-4 days, while in other cases a longer half-life of e.g. about 6-7 days will be desired. As will be explained in further detail below, the number and size of such polymer molecules may be adapted in order to obtain a desired clearance, as well as other desired properties, suitable for a given clinical indication. Preferably, the conjugate of the invention has a reduced clearance of at least about 50%, such as least about 75% or at least about 90%, as compared to the corresponding non-conjugated polypeptide (such as hFSH or rhFSH) as determined under comparable conditions.

Generally, activation of the receptor is coupled to receptor-mediated clearance (RMC) such that binding of a polypeptide to its receptor without activation does not lead to RMC, while activation of the receptor leads to RMC. The clearance is due to internalisation of

the receptor-bound polypeptide with subsequent lysosomal degradation. Reduced RMC may therefore be achieved by designing the conjugate so as to be able to bind and activate a sufficient number of receptors to obtain optimal *in vivo* biological response and avoid activation of more receptors than required for obtaining such response, e.g. by substitution, polymer conjugation or other modification of one or more amino acid residues located at or near a receptor-binding site. This may be reflected in reduced *in vitro* bioactivity and/or increased off-rate.

The term “FSH- α ” is intended to indicate a polypeptide having qualitatively similar functions or activities as the corresponding wildtype FSH α subunit, including the capability of forming a dimeric polypeptide with an FSH- β subunit (FSH- β), which dimeric polypeptide exhibits FSH activity. Alternatively used terms include “FSH- α polypeptide”, “FSH- α subunit”, and “modified FSH- α ”. Analogously, the term “FSH- β ” is intended to indicate a polypeptide having qualitatively similar functions or activities as the corresponding wildtype FSH β subunit, including the capability of dimerizing with FSH- α and thereby forming a dimeric polypeptide exhibiting FSH activity. Alternatively used terms include “FSH- β polypeptide”, “FSH- β subunit”, and “modified FSH- β ”.

The term “exhibiting FSH activity” is intended to indicate that the conjugate or polypeptide has one or more of the functions of wildtype FSH, in particular hFSH, including the capability of binding to and activating an FSH receptor. The FSH activity is conveniently assayed using the *in vitro* activity assay described in the Examples section below. The conjugate or polypeptide “exhibiting” FSH activity is considered to have such activity when it displays a measurable function, e.g. a measurable activity. The dimeric polypeptide exhibiting FSH activity may also be termed “FSH molecule” herein.

Conjugate of the invention

As stated above, in a first aspect the invention relates to a polypeptide conjugate exhibiting FSH activity, comprising i) a polypeptide comprising FSH- α and FSH- β subunits, wherein at least one of the FSH- α and FSH- β subunits differs from the corresponding wildtype subunit in at least one introduced or removed amino acid residue comprising an attachment group for non-polypeptide moiety, and ii) a non-polypeptide moiety bound to an attachment group of the polypeptide. Examples of amino acid residues that may be introduced and/or removed are described in further detail in the following sections.

By removing and/or introducing an amino acid residue comprising an attachment group for the non-polypeptide moiety, it is possible to specifically adapt the polypeptide so as to make the molecule more susceptible to conjugation to the non-polypeptide moiety of choice, to optimize the conjugation pattern (e.g. to ensure an optimal distribution of non-polypeptide moieties on the surface of the FSH molecule and to ensure that only the attachment groups intended to be conjugated are present in the molecule) and thereby obtain a new conjugate molecule which has FSH activity and in addition one or more improved properties as compared to FSH molecules available today, in particular increased functional *in vivo* half-life and/or reduced clearance.

In the conjugate of the invention, one or both of the FSH subunits may be modified according to the invention. For instance, the amino acid sequence of FSH- α may be modified as described herein, whereas FSH- β is unmodified, and vice versa. Alternatively, both of FSH- α and FSH- β may be modified according to the invention.

While the FSH- α and/or FSH- β may be of any origin, it is in particular of mammalian origin, and preferably of human origin. Accordingly, the corresponding wildtype subunits referred to above are preferably hFSH- α and hFSH- β , respectively, with the amino acid sequences shown in SEQ ID NO:2 and 4.

In a preferred embodiment one difference between the amino acid sequence of FSH- α and/or FSH- β and the corresponding wildtype sequence is that at least one and preferably more, e.g. 1-20, amino acid residues comprising an attachment group for the non-polypeptide moiety have been introduced, by insertion or substitution, into the amino acid sequence. Thereby, properties such as the molecular weight, shape, size and/or charge of the conjugate can be optimised. Preferably, such amino acid residues are introduced in positions occupied by an amino acid residue having more than 25%, more preferably more than 50%, such as more than 75% of its side chain exposed at the surface of the molecule.

The term "one difference" as used in the present application is intended to allow for additional differences being present. Accordingly, in addition to the specified amino acid difference, other amino acid residues than those specified may be mutated.

In one embodiment, one difference between the amino acid sequence of FSH- α and/or FSH- β and that of the corresponding wildtype polypeptide is that at least one and possibly more, e.g. 1-15, amino acid residues comprising an attachment group for the non-polypeptide moiety have been removed, by substitution or deletion, from the amino acid sequence. The amino acid residue to be removed is preferably one to which conjugation is dis-

advantageous, e.g. an amino acid residue located at or near a functional site of the polypeptide (since conjugation at such a site may result in inactivation or reduced FSH activity of the resulting conjugate due to impaired receptor recognition). In the present context the term “functional site” is intended to indicate one or more amino acid residues which are essential for or otherwise involved in the function or performance of hFSH, in particular dimerization and/or receptor binding and activation. Such amino acid residues are a part of a functional site. The functional site may be determined by methods known in the art and is preferably identified by analysis of a structure of the polypeptide complexed to a relevant receptor, such as the hFSH receptor.

In another embodiment, the alteration of FSH- α and/or FSH- β embraces removal as well as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice.

In order to avoid too much disruption of the structure and function of the FSH molecule, the total number of amino acid residues to be altered in accordance with the present invention will typically not exceed 20 for each individual subunit. Preferably, the polypeptide part of the conjugate of the invention or the dimeric polypeptide of the invention comprises an amino acid sequence which differs in a total of 1-20 amino acid residues from the amino acid sequences shown in SEQ ID NO:2 and/or SEQ ID NO:4, such as in 1-15 or 2-12 amino acid residues, e.g. in 3-10 amino acid residues. Thus, normally the polypeptide part of the conjugate or the dimeric polypeptide of the invention comprises an amino acid sequence which in total differs from the amino acid sequences shown in SEQ ID NO:2 and/or SEQ ID NO:4 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid residues.

The FSH- α and/or FSH- β subunits of the dimeric polypeptide are preferably any of the specific modified FSH- α and/or FSH- β polypeptides disclosed in the subsequent sections having introduced and/or removed amino acid residues comprising an attachment group for the relevant non-polypeptide moiety.

The amino acid residue comprising an attachment group for a non-polypeptide moiety, whether it is removed or introduced, is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide and the non-polypeptide moiety is to be achieved. It will be understood that in order to preserve a measurable function of the modified FSH- α and/or FSH- β , amino acid residues to be modified (by deletion or substitution) are selected from those amino acid residues which are not essential for providing a measurable activity. Accord-

ingly, amino acid residues to be modified are different from those required for subunit dimerization and/or receptor binding or activation. The identity of such amino acid residues is described in the art (e.g. references identified in the Background section above) or can be determined by a person skilled in the art using methods known in the art.

5 In addition to the amino acid alterations disclosed herein aimed at introducing and/or removing attachment sites for the non-polypeptide moiety, the FSH- α and/or FSH- β subunits may comprise other amino acid alterations that need not be related to introduction or removal of attachment sites, i.e. other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues, 10 or addition of one or more extra residues at the N- and/or C-terminus. Examples of such additional amino acid changes include adding part of or the entire CTP region of hCG to the C-terminus of FSH- α or introducing any other mutation (in particular selected among those reported to enhance FSH activity and/or increase the functional *in vivo* half-life, cf. the Background of the Invention section herein). In such cases, the amino acid sequence of the basic 15 polypeptide subunits, i.e. the sequence of the subunits excluding any introduced or removed attachment sites, will typically have a degree of homology, compared to the relevant wildtype sequence (normally hFSH- α or hFSH- β), of at least about 80%, more typically at least about 90%, such as at least about 95%. Amino acid sequence homology/identity is conveniently determined from aligned sequences, using e.g. the ClustalW program or from the PFAM families database version 4.0 (<http://pfam.wustl.edu/>) (*Nucleic Acids Res.* 1999 Jan 1; 27(1):260-2) 20 by use of GENEDOC version 2.5 (Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K.B. and Nicholas H.B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

25 Preferably, the conjugate of the present invention has one or more improved properties as compared to hFSH, including increased functional *in vivo* half-life, increased serum half-life, reduced renal clearance, reduced receptor-mediated clearance, reduced immunogenicity and/or an increased bioavailability as compared to rhFSH (e.g. Gonal-F® or Puregon®). Consequently, medical treatment with a conjugate of the invention offers advantages 30 over the currently available FSH compounds, in particular longer duration between injections.

Conjugate of the invention wherein the non-polypeptide moiety is an oligosaccharide moiety

It has been found that N-glycosylation is important for FSH activity and also that the extent and type of oligosaccharide moiety attached by *in vivo* glycosylation is important for functional *in vivo* half-life of the glycosylated FSH. In order to obtain a different, increased glycosylation it is desirable to introduce at least one glycosylation site. Accordingly, in a preferred aspect the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity comprising a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein the amino acid sequence of at least one of the FSH- α and FSH- β subunits differs from that of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced, and having at least one oligosaccharide moiety bound to an N-glycosylation site of at least one of the subunits.

A suitable N-glycosylation site may be introduced by introducing, by substitution or insertion, an asparagine residue in a position occupied by an amino acid residue having more than 25% of its side chain exposed at the surface of the polypeptide, which position does not have a proline residue located in position +1 or +3 therefrom. If the amino acid residue located in position +2 is a serine or threonine, no further amino acid substitution is required. However, if this position is occupied by a different amino acid residue, a serine or threonine residue needs to be introduced.

A preferred conjugate according to this embodiment is one which comprises a modified FSH- α subunit having an amino acid residue which differs from that of hFSH- α in the introduction of at least one N-glycosylation site by means of a mutation selected from the group consisting of P2(a)N+V4(a)S, P2(a)N+V4(a)T, D3(a)N+Q5(a)S, D3(a)N+Q5(a)T, V4(a)N+D6(a)S, V4(a)N+D6(a)T, D6(a)N+P8(a)S, D6(a)N+P8(a)T, E9(a)N+T11(a)S, E9(a)N, T11(a)N+Q13(a)S, T11(a)N+Q13(a)T, L12(a)N+E14(a)S, L12(a)N+E14(a)T, E14(a)N+P16(a)S, E14(a)N+P16(a)T, P16(a)N+F18(a)S, P16(a)N+F18(a)T, F17(a)N, F17(a)N+S19(a)T, G22(a)N+P24(a)S, G22(a)N+P24(a)T, P24(a)N+L26(a)S, P24(a)N+L26(a)T, F33(a)N+R35(a)S, F33(a)N+R35(a)T, R42(a)N+K44(a)S, R42(a)N+K44(a)T, S43(a)N+K45(a)S, S43(a)N+K45(a)T, K44(a)N+T46(a)S, K44(a)N, K45(a)N+M47(a)S, K45(a)N+M47(a)T, T46(a)N+L48(a)S, T46(a)N+L48(a)T, L48(a)N+Q50(a)S, L48(a)N+Q50(a)T, V49(a)N+K51(a)S, V49(a)N+K51(a)T, Q50(a)N+N52(a)S, Q50(a)N+N52(a)T, V61(a)N+K63(a)S, V61(a)N+K63(a)T, K63(a)N+Y65(a)S, K63(a)N+Y65(a)T, S64(a)N+N66(a)S, S64(a)N+N66(a)T, Y65(a)N+R67(a)S, Y65(a)N+R67(a)T, V68(a)S, V68(a)T, R67(a)N+T69(a)S, R67(a)N,

T69(a)N+M71(a)S, T69(a)N+M71(a)T, M71(a)N+G73(a)S, M71(a)N+G73(a)T, G72(a)N+F74(a)S, G72(a)N+F74(a)T, G73(a)N+K75(a)S, G73(a)N+K75(a)T, F74(a)N+V76(a)S, F74(a)N+V76(a)T, K75(a)N+E77(a)S, K75(a)N+E77(a)T, A81(a)N+H83(a)S, A81(a)N+H83(a)T, H83(a)N, T86(a)N+Y88(a)S, T86(a)N+Y88(a)T, Y88(a)N+H90(a)S, Y88(a)N+H90(a)T, Y89(a)N+K91(a)S, Y89(a)N+K91(a)T, H90(a)N and H90(a)N+S92(a)T (positions with more than 25% side chain exposure). Among these possible positions for mutation, more preferred mutations are those where a glycosylation site can be introduced by mutation of a single amino acid residue, i.e. selected from the group consisting of V68(a)S, V68(a)T, E9(a)N, F17(a)N, K44(a)N, R67(a)N, H83(a)N and H90(a)N.

More preferably, a glycosylation site is introduced at a position having more than 50% side chain exposure, i.e. by means of a mutation selected from the group consisting of P2(a)N+V4(a)S, P2(a)N+V4(a)T, D3(a)N+Q5(a)S, D3(a)N+Q5(a)T, V4(a)N+D6(a)S, V4(a)N+D6(a)S, D6(a)N+P8(a)S, D6(a)N+P8(a)T, E9(a)N+T11(a)S, E9(a)N, T11(a)N+Q13(a)S, T11(a)N+Q13(a)T, E14(a)N+P16(a)S, E14(a)N+P16(a)T, P16(a)N+F18(a)S, P16(a)N+F18(a)T, F17(a)N, F17(a)N+S19(a)T, G22(a)N+P24(a)S, G22(a)N+P24(a)T, K45(a)N+M47(a)S, K45(a)N+M47(a)T, T46(a)N+L48(a)S, T46(a)N+L48(a)T, L48(a)N+Q50(a)S, L48(a)N+Q50(a)T, V49(a)N+K51(a)S, V49(a)N+K51(a)T, Q50(a)N+N52(a)S, Q50(a)N+N52(a)T, K63(a)N+Y65(a)S, K63(a)N+Y65(a)T, S64(a)N+N66(a)S, S64(a)N+N66(a)T, V68(a)S, V68(a)T, R67(a)N+T69(a)S, R67(a)N, T69(a)N+M71(a)S, T69(a)N+M71(a)T, G72(a)N+F74(a)S, G72(a)N+F74(a)T, G73(a)N+K75(a)S, G73(a)N+K75(a)T, K75(a)N+E77(a)S, K75(a)N+E77(a)T, T86(a)N+Y88(a)S, T86(a)N+Y88(a)T, Y89(a)N+K91(a)S, Y89(a)N+K91(a)T, H90(a)N, and H90(a)N+S92(a)T. Still more preferably, glycosylation sites are introduced via mutation of a single amino acid residue selected from the group consisting of E9(a)N, F17(a)N, R67(a)N, and H90(a)N.

The FSH- β part of such conjugates with an altered FSH- α subunit may be hFSH- β or any of the modified FSH- β polypeptides described herein.

Alternatively or additionally, the conjugate according to this embodiment comprises a modified FSH- β having an amino acid residue which differs from that of hFSH- β in the introduction of at least one N-glycosylation site by a mutation selected from the group consisting of S2(b)N+E4(b)S, S2(b)N+E4(b)T, E4(b)N+T6(b)S, E4(b)N, L5(b)N+N7(b)S, L5(b)N+L7(b)T, T6(b)N+I8(b)S, T6(b)N+I8(b)T, I8(b)N+I10(b)S, I8(b)N+I10(b)T, T9(b)N+A11(b)S, T9(b)N+A11(b)T, K14(b)N+E16(b)S, K14(b)N+E16(b)T,

F19(b)N+I21(b)S, F19(b)N+I21(b)T, I21(b)N+I23(b)S, I21(b)N+I23(b)T, S22(b)N+N24(b)S, S22(b)N+N24(b)T, Y31(b)N+Y33(b)S, Y31(b)N+Y33(b)T, Y33(b)N+R35(b)S, Y33(b)N+R35(b)T, R35(b)N+L37(b)S, R35(b)N+L37(b)T, D36(b)N+V38(b)S, D36(b)N+V38(b)T, L37(b)N+Y39(b)S, L37(b)N+Y39(b)T, K40(b)N+P42(b)S, K40(b)N+P42(b)T, A43(b)N+P45(b)S, A43(b)N+P45(b)T, P45(b)N+I47(b)S, P45(b)N+I47(b)T, K46(b)N+Q48(b)S, K46(b)N+Q48(b)T, I47(b)N+K49(b)S, I47(b)N+K49(b)T, K54(b)N+L56(b)S, K54(b)N+L56(b)T, E55(b)N+V57(b)S, E55(b)N+V57(b)T, L56(b)N+Y58(b)S, L56(b)N+Y58(b)T, V57(b)N+E59(b)S, V57(b)N+E59(b)T, Y58(b)N+T60(b)S, Y58(b)N, E59(b)N+V61(b)S, E59(b)N+V61(b)T, T60(b)N+R62(b)S, T60(b)N+R62(b)T, R62(b)N+P64(b)S, R62(b)N+P64(b)T, G65(b)N+A67(b)S, G65(b)N+A67(b)T, A67(b)N+H69(b)S, A67(b)N+H69(b)T, H68(b)N+A70(b)S, H68(b)N+A70(b)T, H69(b)N+D71(b)S, H69(b)N+D71(b)T, D71(b)N+L73(b)S, D71(b)N+L73(b)T, L73(b)N+T75(b)S, L73(b)N, T75(b)N+P77(b)S, T75(b)N+P77(b)T, H83(b)N+G85(b)S, H83(b)N+G85(b)T, K86(b)N+D88(b)S, K86(b)N+D88(b)T, D88(b)N+D90(b)S, D88(b)N+D90(b)T, S89(b)N, S89(b)N+S91(b)T, D90(b)N+T92(b)S, D90(b)N, S91(b)N+D93(b)S, S91(b)N+D93(b)T, D93(b)N+T96(b)S, D93(b)N, T95(b)N+R97(b)S, T95(b)N+R97(b)T, V96(b)N+G98(b)S, V96(b)N+G98(b)T, R97(b)N+L99(b)S, R97(b)N+L99(b)T, L99(b)N+P101(b)S, L99(b)N+P101(b)T, Y103(b)N, Y103(b)N+S105(b)T, S105(b)N+G107(b)S, S105(b)N+G107(b)T, F106(b)N+E108(b)S, F106(b)N+E108(b)T, G107(b)N+M109(b)S, G107(b)N+M109(b)T, E108(b)N+K110(b)S, E108(b)N+K110(b)T, M109(b)N+E111(b)S, and M109(b)N+E111(b)T (mutations at positions with at least 25% side chain exposure). Preferably, glycosylation sites are introduced by means of mutation of a single amino acid residue selected from the group consisting of E4(b)N, Y58(b)N, L73(b)N, S89(b)N, D90(b)N, D93(b)N, and Y103(b)N.

More preferably, a modified FSH- β has an amino acid residue which differs from that of hFSH- β in the introduction of at least one N-glycosylation site by a mutation selected from the group consisting of F19(b)N+I21(b)S, F19(b)N+I21(b)T, Y33(b)N+R35(b)S, Y33(b)N+R35(b)T, A43(b)N+P45(b)S, A43(b)N+P45(b)T, P45(b)N+I47(b)S, P45(b)N+I47(b)T, K46(b)N+Q48(b)S, K46(b)N+Q48(b)T, I47(b)N+K49(b)S, I47(b)N+K49(b)T, K54(b)N+L56(b)S, K54(b)N+L56(b)T, E55(b)N+V57(b)S, E55(b)N+V57(b)T, V57(b)N+E59(b)S, V57(b)N+E59(b)T, Y58(b)N+T60(b)S, Y58(b)N, E59(b)N+V61(b)S, E59(b)N+V61(b)T, R62(b)N+P64(b)S, R62(b)N+P64(b)T, G65(b)N+A67(b)S, G65(b)N+A67(b)T, A67(b)N+H69(b)S, A67(b)N+H69(b)T,

H68(b)N+A70(b)S, H68(b)N+A70(b)T, H69(b)N+D71(b)S, H69(b)N+D71(b)T, D71(b)N+L73(b)S, D71(b)N+L73(b)T, L73(b)N+T75(b)S, L73(b)N, T75(b)N+P77(b)S, T75(b)N+P77(b)T, H83(b)N+G85(b)S, H83(b)N+G85(b)T, K86(b)N+D88(b)S, K86(b)N+D88(b)T, D88(b)N+D90(b)S, D88(b)N+D90(b)T, S89(b)N, S89(b)N+S91(b)T, 5 D90(b)N+T92(b)S, D90(b)N, S91(b)N+D93(b)S, S91(b)N+D93(b)T, T95(b)N+R97(b)S, T95(b)N+R97(b)T, R97(b)N+L99(b)S, R97(b)N+L99(b)T, L99(b)N+P101(b)S, L99(b)N+P101(b)T, Y103(b)N, Y103(b)N+S105(b)T, S105(b)N+G107(b)S, S105(b)N+G107(b)T, F106(b)N+E108(b)S, F106(b)N+E108(b)T, G107(b)N+M109(b)S, G107(b)N+M109(b)T, E108(b)N+K110(b)S, E108(b)N+K110(b)T, M109(b)N+E111(b)S, 10 and M109(b)N+E111(b)T (positions having more than 50% side chain accessibility). Among these positions, it is preferred to introduce glycosylation sites using mutation of a single amino acid residue selected from the group consisting of Y58(b)N, L73(b)N, S89(b)N, D90(b)N, and Y103(b)N.

The FSH- α part of such conjugates with an altered FSH- β subunit may be 15 hFSH- α or any of the modified FSH- α polypeptides described herein.

The FSH- α and/or FSH- β polypeptide may further differ from hFSH- α and/or hFSH- β in at least one removed, naturally occurring N-glycosylation site. In particular, FSH- α may comprise a substitution of N78(a) and/or T80(a) by any other amino acid residue and/or FSH- β may comprise a substitution of N7(b), T9(b), N24(b) and/or T26(b) by any 20 other amino acid residue. Preferably, the N residue is substituted by Q or D, and the T residue by A or G.

Furthermore, one or both of the FSH- α and FSH- β subunits of the conjugate according to this embodiment (having at least one of the above mentioned N-glycosylation site modifications) may differ from hFSH- α and hFSH- β , respectively, in the removal, pref- 25 erably by substitution, of at least one lysine residue. See the section below on removal of lysine residues for further details.

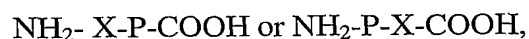
An alternative embodiment of this aspect of the invention is one in which at least one of said FSH- α and FSH- β subunits comprises at least one introduced N- or O-glycosylation site at the N-terminal thereof, and wherein the at least one introduced glycosyla- 30 tion site is glycosylated; see the discussion of peptide addition below. In this case, the respective subunits may comprise one or more of the modifications disclosed elsewhere herein, or one or both of the subunits may be the respective wildtype subunits, but having the at least one introduced terminal glycosylation site. Thus, the polypeptide conjugate may be one in

which the FSH- α subunit comprises hFSH- α having the sequence shown in SEQ ID NO:2, and/or in which the FSH- β subunit comprises hFSH- β having the sequence shown in SEQ ID NO:4. In a particular embodiment, both of the subunits correspond to the respective wildtype hFSH subunits, although with either the α or β subunit, or both, having an introduced N-terminal glycosylation site.

The introduced glycosylation site may be of the type described elsewhere herein; see the discussion of glycosylation under the general discussion of attachment groups above. A non-limiting example of a suitable glycosylation site for introduction at the N-terminal is the sequence Ala-Asn-Ile-Thr-Val-Asn-Ile-Thr-Val, e.g. for insertion of two glycosylation sites upstream of a mature FSH- α or FSH- β sequence.

Introduction of glycosylation sites by means of peptide addition

In addition to or as an alternative to introducing glycosylation sites within the amino acid sequence of one or both of the subunits, one or more additional glycosylation sites may be introduced by means of a "peptide addition" as discussed in the following. In this case, each of the polypeptide subunits comprises or consists of or consists essentially of the primary structure,



wherein

X is a peptide addition comprising or contributing to a glycosylation site, and P is the basic polypeptide subunit to be modified, i.e. FSH- α or FSH- β , e.g. a wildtype polypeptide subunit as defined herein or a modified polypeptide having introduced and/or removed glycosylation sites or other attachment sites in the mature part of the polypeptide.

In the context of a peptide addition the term "comprising a glycosylation site" is intended to mean that a complete glycosylation site is present in the peptide addition, whereas the term "contributing to a glycosylation site" is intended to cover the situation where at least one amino acid residue of an N-glycosylation site is present in the peptide addition while the other amino acid residue of said site is present in the polypeptide P, whereby the glycosylation site can be considered to bridge the peptide addition and the polypeptide.

Usually, the peptide addition is fused to the N-terminal or C-terminal end of the polypeptide P as reflected in the above shown structure so as to provide an N- or C-terminal elongation of the polypeptide P, preferably at the N-terminal. However, it is also possible to insert the peptide addition within the amino acid sequence of the polypeptide P whereby the

polypeptide comprises, consists of or consists essentially of the primary structure $\text{NH}_2\text{-P}_x\text{-X-P}_y\text{-COOH}$, wherein

P_x is an N-terminal part of the relevant polypeptide P,

P_y is a C-terminal part of said polypeptide P, and

5 X is a peptide addition comprising or contributing to a glycosylation site.

In order to minimize structural changes effected by the insertion of the peptide addition within the sequence of the polypeptide P, it is desirable that it be inserted in a non-structural part thereof. For instance, P_x may be a non-structural N-terminal part of a mature polypeptide P, and P_y a structural C-terminal part of said mature polypeptide, or P_x may be a
10 structural N-terminal part of a mature polypeptide P, and P_y a non-structural C-terminal part of said mature polypeptide.

The term "non-structural part" is intended to indicate a part of either the C- or N-terminal end of the folded polypeptide subunit that is outside the first structural element, such as an α -helix or a β -sheet structure. The non-structural part can easily be identified in
15 a three-dimensional structure or model of the polypeptide. If no structure or model is available, a non-structural part typically comprises or consists of the first or last 1-20 amino acid residues, such as 1-10 amino acid residues of the amino acid sequence constituting the mature form of the polypeptide.

When the peptide addition comprises only few amino acid residues, e.g. 1-5,
20 such as 1-3 amino acid residues, and in particular one amino acid residue, the peptide addition can be inserted into a loop structure of the polypeptide P and thereby elongate the loop.

In principle, the peptide addition X can be any stretch of amino acid residues ranging from a single amino acid residue to a mature protein. In the present context, it is contemplated that each peptide addition will normally comprise up to about 50 amino acid residues, such as 2-30 or 3-20 amino acid residues. The peptide addition may be designed by a
25 site-specific or random approach. In order to minimize the risk of an immunogenic response, however, it is preferable to select N- or C-terminal extensions of the FSH sequence that comprise peptide sequences that are part of naturally occurring human proteins. Non-limiting examples of such peptide sequences include the sequence NSTQNATA, which corresponds to
30 positions 231 to 238 of the human calcium activated channel 2 precursor (to add two N-glycosylation sites to FSH), or the sequence ANLTVRNLTRNVTV, which corresponds to positions 538 to 551 of the human G protein coupled receptor 64 (to add three N-glycosylation sites to FSH).

Typically, each peptide addition X comprises 1-10 glycosylation sites. The peptide addition X may thus comprise 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 glycosylation sites. It is well known that a frequently occurring consequence of modifying an amino acid sequence of, e.g., a human protein is that new epitopes are created by such modification. Non-polypeptide moieties may be used to shield any new epitopes created by the peptide addition, and therefore it is desirable that sufficient glycosylation sites (or attachment groups for another non-polypeptide moiety, e.g. a polymer such as PEG) are present to enable shielding of all epitopes introduced into the sequence. This is e.g. achieved when the peptide addition X comprises at least one glycosylation site within a stretch of 30 contiguous amino acid residues, preferably as at least one glycosylation sites within 20 amino acid residues, more preferably at least one attachment group within 10 amino acid residues, in particular 1-3 attachment groups within a stretch of 10 contiguous amino acid residues in the peptide addition X.

Preferably, the glycosylation site of the peptide addition is an *in vivo* glycosylation site, preferably an N-glycosylation site. For instance, the peptide addition X may have the structure $X_1\text{-N-X}_2\text{-T/S/C-Z}$, wherein X_1 is a peptide comprising at least one amino acid residue or is absent, X_2 is any amino acid residue different from P, and Z is absent or is a peptide comprising at least one amino acid residue. For instance, X_1 may be absent, X_2 may be an amino acid residue selected from the group consisting of I, A, G, V and S (all relatively small amino acid residues), and Z may comprise at least 1 amino acid residue. Z can e.g. be a peptide comprising up to 50 amino acid residues and e.g. up to 10 glycosylation sites.

Alternatively, X_1 may comprise at least one amino acid residue, e.g. 1-50 amino acid residues with 1-10 glycosylation sites, X_2 may be an amino acid residue selected from the group consisting of I, A, G, V and S, and Z may be absent.

Examples of peptide additions for use in the present invention are ANITVNITV, NDTVNFT and NITVNITV; see Examples 9 and 10 below, which illustrate addition of these sequences at the N-terminal of the FSH- α and β subunits.

The peptide addition can comprise one or more of these peptide sequences, i.e. at least two of said sequences either directly linked together or separated by one or more amino acid residues, or can contain two or more copies of any of these peptide sequence. It will be understood that the above specific sequences are given for illustrative purposes and thus do not constitute an exhaustive list of peptide sequences of use in the present invention.

In one embodiment, the peptide addition X has an N residue in position -2 or -1, and the polypeptide P or P_x has a T or an S residue in position +1 or +2, respectively, the

residue numbering being made relative to the N-terminal amino acid residue of P or P_x, whereby an N-glycosylation site is formed. For instance, the polypeptide may have a T or S residue in position 2, preferably a T residue, and the peptide addition is AN or comprises AN as the C-terminal amino acid residues.

5

O-glycosylation

As an alternative or in addition to the mutations discussed above, the heterodimeric polypeptide may comprise one or more introduced O-glycosylation sites, for example the amino acid sequence AATPAP, which has been found to be an efficient signal sequence for O-glycosylation *in vivo* (Asada et al. (1999) *Glycoconj. J.* 16(7):321-6). The AATPAP sequence for O-glycosylation is preferably introduced by way of insertion at the N- and/or C-terminus of the FSH- α and/or FSH- β subunit.

10

Preparation of glycosylated conjugates

15

It will be understood that in order to prepare a conjugate according to this aspect, the polypeptide must be expressed in a glycosylating host cell capable of attaching oligosaccharide moieties at the glycosylation site(s) *in vivo* or alternatively subjected to *in vitro* glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to an oligosaccharide moiety".

20

In addition to an oligosaccharide moiety, the conjugate according to the aspect of the invention described in the present section may contain additional non-polypeptide moieties different from O-linked or N-linked oligosaccharide moieties, in particular a polymer molecule such as PEG as described herein conjugated to one or more attachment groups present in the polypeptide part of the conjugate. This is particularly relevant when a lysine residue (or any other amino acid residue comprising an attachment group for the polymer molecule in question) has been introduced and/or removed.

25

It will be understood that any of the amino acid changes specified in this section can be combined with any of the amino acid changes specified in the other sections herein disclosing specific amino acid changes.

30

Conjugate of the invention wherein the non-polypeptide moiety is attached to a lysine or the N-terminal amino acid residue

In a further preferred embodiment the conjugate of the invention is one wherein the amino acid residue comprising an attachment group for the non-polypeptide moiety is a lysine residue and the non-polypeptide moiety is any molecule which has lysine as an attachment group. For instance, the non-polypeptide moiety may be a polymer molecule, in particular any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule", and preferably selected from the group consisting of linear or branched polyethylene glycol and polyalkylene oxide. Most preferably, the polymer molecule is mPEG-SPA or oxy-carbonyl-oxy-N-dicarboxyimide PEG (US 5,122,614).

The FSH- α and/or FSH- β having introduced and/or removed at least one lysine may advantageously be *in vivo* glycosylated, e.g. using naturally occurring glycosylation sites present in the relevant FSH polypeptide. However, in a particular embodiment the conjugate is one wherein the amino acid sequence of FSH- α and/or FSH β differs from that of FSH- α and/or FSH- β in that an N-glycosylation site has been introduced and/or removed. Such introduced/removed sites may be any of those described in the section entitled "Conjugate of the invention wherein the non-polypeptide moiety is an oligosaccharide moiety".

i) Removal of lysine residues

hFSH- α contains 6 lysine residues and hFSH- β 7. In order to avoid conjugation to one or more of these lysine residues, e.g. lysine residues located at or close to the receptor-binding site of hFSH, it may be desirable to remove at least one lysine residue. Accordingly, in one embodiment the conjugate of the invention is one which comprises a modified FSH- α having an amino acid residue which differs from that of hFSH- α in the removal of at least one lysine residue selected from the group consisting of K44(a), K45(a), K51(a), K63(a), K75(a), and K91(a), in particular at least one amino acid residue selected from of the group consisting of K44(a), K45(a), K63(a), K75(a), and K91(a) (these residues having more than 25% of their side chain exposed to the surface), and preferably from the group consisting of K45(a), K63(a), K75(a), and K91(a) (these residues having more than 50% of their side chain exposed to the surface). The FSH- β part of this conjugate may be hFSH- β or any of the modified FSH- β polypeptides described herein.

In another embodiment the conjugate of the invention is one which comprises a modified FSH- β having an amino acid residue which differs from that of hFSH- β in the removal of at least one lysine residue selected from the group consisting of K14(b), K40(b), K46(b), K49(b), K54(b), K86(b), and K110(b), in particular at least one amino acid residue
5 selected from of the group consisting of K14(b), K40(b), K46(b), K49(b), K54(b), K86(b), and K110(b) (these residues having more than 25% of their side chain exposed to the surface), and preferably from the group consisting of K46(b), K54(b), K86(b), and K110(b) (these residues having more than 50% of their side chain exposed to the surface). The FSH- α part of this conjugate may be hFSH- α or any of the modified FSH- α polypeptides described herein.

10 In a further embodiment, the conjugate of the invention is one which comprises a modified FSH- α and a modified FSH- β , each of which differ from the corresponding hFSH subunit in the removal of at least one of the above identified lysine residues. For instance, the conjugate of the invention may be one wherein the modified FSH- α and modified FSH- β subunit differ from the corresponding hFSH subunit in at least one of K45(a), K63(a), K75(a),
15 and K91(a) and at least one of K46(b), K54(b), K86(b), and K110(b).

The removal of any of the above lysine residues is preferably achieved by substitution by any other amino acid residue, in particular by an arginine or a glutamine residue.

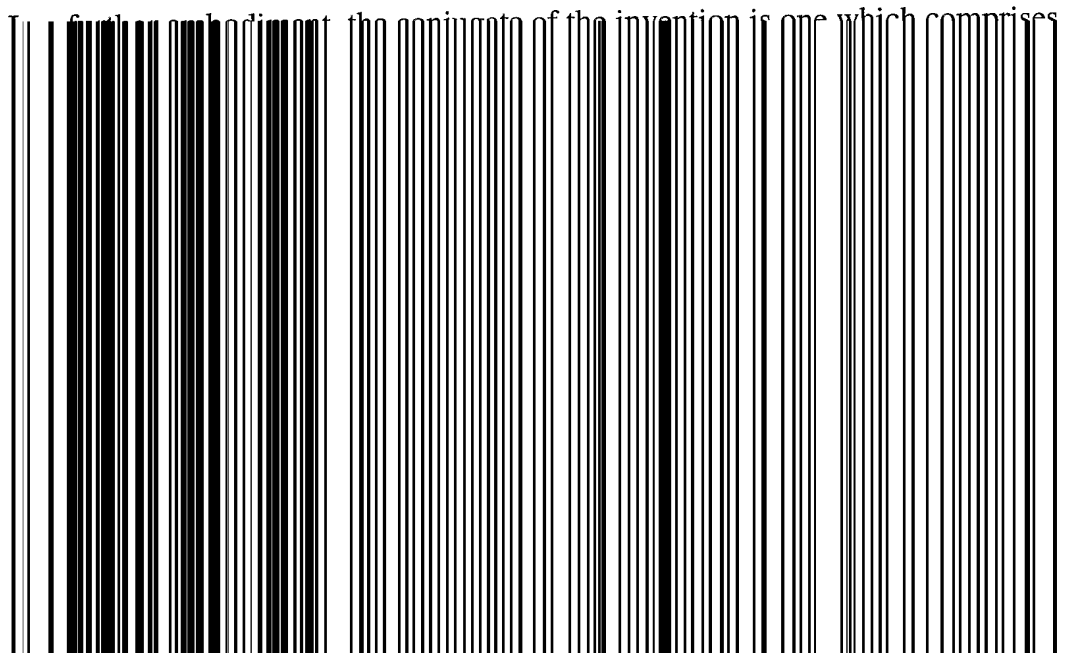
ii) Introduction of lysine residues

20 In order to obtain a more extensive conjugation it may be desirable to introduce at least one non-naturally occurring lysine residue in hFSH, in particular in a position occupied by an amino acid residue having a side chain which is more than 25% surface exposed and which is not part of a cystine or located at a receptor binding site.

Accordingly, in a further embodiment the conjugate of the invention is one
25 which comprises a modified FSH- α having an amino acid residue which differs from that of hFSH- α in the introduction of at least one lysine residue in a position selected from the group consisting of A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), L12(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), P24(a), L26(a), M29(a), F33(a), R42(a), S43(a), T46(a), L48(a), V49(a), Q50(a), N52(a), V61(a), S64(a), Y65(a), N66(a),
30 R67(a), V68(a), T69(a), M71(a), G72(a), G73(a), F74(a), N78(a), T80(a), A81(a), H83(a), S85(a), T86(a), Y88(a), Y89(a), H90(a), and S92(a), in particular selected from of the group consisting of A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), Q13(a), E14(a),

P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), T46(a), L48(a), V49(a), Q50(a), N52(a), S64(a), N66(a), R67(a), T69(a), G72(a), G73(a), T86(a), Y89(a), H90(a), and S92(a) (these residues having more than 50% of their side chain exposed to the surface), and most preferably in the position R42(a) and/or R67(a), such as R67(a). The FSH- β part of this conjugate
 5 may be hFSH- β or any of the modified FSH- β polypeptides described herein.

In a further embodiment the conjugate of the invention is one which comprises a modified FSH- β having an amino acid residue which differs from that of hFSH- β in the introduction of at least one lysine residue in a position selected from the group consisting of N1(b), S2(b), E4(b), L5(b), T6(b), N7(b), I8(b), T9(b), E15(b), E16(b), R18(b), F19(b),
 10 I21(b), S22(b), N24(b), Y31(b), Y33(b), R35(b), D36(b), L37(b), Y39(b), D41(b), P42(b), A43(b), R44(b), P45(b), I47(b), E55(b), L56(b), V57(b), Y58(b), E59(b), T60(b), V61(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), Y74(b), T75(b), T80(b), Q81(b), H83(b), G85(b), D88(b), S89(b), D90(b), S91(b), D93(b), T95(b), V96(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), and
 15 E111(b), in particular selected from of the group consisting of N1(b), N7(b), T9(b), E15(b), E16(b), R18(b), F19(b), N24(b), Y33(b), D41(b), P42(b), A43(b), R44(b), P45(b), I47(b), E55(b), V57(b), Y58(b), E59(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), T75(b), Q81(b), H83(b), D88(b), S89(b), D90(b), S91(b), T95(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), and E111(b) (these
 20 residues having more than 50% of their side chain exposed to the surface), and most preferably selected from the group consisting of R18(b), R35(b), R44(b), R62(b), and R97(b), such as R18(b), R44(b), R62(b), and R97(b). The FSH- α part of this conjugate may be hFSH- α or any of the modified FSH- α polypeptides described herein.



iii) Introduction and removal of lysine residues

The conjugate of the invention may comprise at least one introduced lysine residue, in particular any of those described in the section entitled "Introduction of lysine residues", and at least one removed lysine residue, in particular any of those described in the section entitled "Removal of lysine residues".

Preferably, the conjugate comprises a modified FSH- α and/or a modified FSH- β which differs from the corresponding hFSH- α/β in at least one introduced and at least one removed lysine residue, wherein the lysine residue is introduced by substitution of an amino acid residue selected from the group consisting of R42(a) and R67(a), R18(b), R35(b), R44(b), R62(b), and R97(b), and more preferably from the group consisting of R67(a), R18(b), R44(b), R62(b), and R97(b) and removal of a lysine residue selected from the group consisting of K45(a), K63(a), K75(a), K91(a), K46(b), K54(b), K86(b), and K110(b), the removal preferably being achieved by substitution by any other amino acid residue, in particular by an arginine residue.

N-terminal PEGylation of FSH

As indicated above, one aspect of the invention relates to a polypeptide conjugate wherein at least one of the FSH- α and FSH- β subunits comprises a polymer molecule bound to the N-terminal thereof. Preferably, the polymer is a polyethylene glycol (PEG) such as mPEG; see the general discussion below regarding conjugates comprising polyethylene glycol-derived polymers.

In the case of N-terminal PEGylated FSH conjugates according to the invention, the respective subunits may comprise one or more of the modifications disclosed elsewhere herein, or one or both of the subunits may be the respective wildtype subunits with a PEG-derived polymer being attached at the N-terminal. Thus, the polypeptide conjugate may be one in which the FSH- α subunit comprises hFSH- α having the sequence shown in SEQ ID NO:2, and/or in which the FSH- β subunit comprises hFSH- β having the sequence shown in SEQ ID NO:4. In one embodiment, both of the subunits correspond to the respective wildtype hFSH subunits, although with either the α or β subunit, or both, being N-terminally PEGylated. In a preferred embodiment, however, at least one glycosylation site has been introduced into one or both of the subunits as described in detail above. In cases where at least one of the subunits has an N-terminally attached PEG molecule, it will often be desirable that no other

PEG molecules are attached, e.g., to a lysine residue. In such cases, the polypeptide conjugate will thus comprise either one or two N-terminally attached PEG molecules as the sole polymer molecule(s).

Aldehyde-activated PEG and reduction using NaBH_3CN have been used to selectively pegylate the N-terminal α -amino group of proteins (see for instance US 5,824,784 regarding N-terminal PEGylation of G-CSF). The N-terminus of the α and/or the β chain of wildtype FSH or a modified form of FSH can be PEGylated using similar methods. Reaction materials include purified FSH or a modified form of FSH, methoxy-PEG-aldehyde (M-PEG-CHO), and NaBH_3CN . In order to optimise yield, one may for instance vary: molar ratio of FSH, M-PEG-CHO and NaBH_3CN , time for establishment of the Schiff's base equilibrium (reaction between FSH and M-PEG-CHO before addition of NaBH_3CN), reaction time after addition of NaBH_3CN , temperature, pH, or reaction volume. The yield of PEGylated FSH forms may be analysed using Western blotting, mass spectrometry and N-terminal sequencing. In order to restrict PEGylation to only one of the two N-termini in FSH, PEGylation of the α or β chain may be selectively prevented by addition of a glutamine to the N-terminus. Spontaneous cyclisation of such an N-terminal glutamine residue will render it inaccessible for PEGylation. Such a glutamine residue may subsequently be removed using a pyroglutamyl aminopeptidase (for instance EC 3.4.19.3).

Conjugate of the invention having a non-lysine residue as an attachment group

Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups may be introduced into and/or removed from FSH- α and/or FSH- β , using the same approach as that illustrated above by lysine residues. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), asparagine, tyrosine or cysteine may be introduced into positions which in hFSH are occupied by amino acid residues having surface exposed side chains (i.e. the positions mentioned above as being of interest for introduction of lysine residues), or removed. As described above, introduction or removal of such amino acid residues is preferably performed by substitution. Preferably, Asp is substituted by Asn, Glu by Gln, Tyr by Phe, and Cys by Ser. Another possibility is introduction and/or removal of a histidine, e.g. by substitution with arginine.

Non-polypeptide moiety of the conjugate of the invention

As indicated above, the non-polypeptide moiety of the conjugate of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, an oligosaccharide moiety (by way of *in vivo* glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the polypeptide part of the conjugate, in particular an increased functional *in vivo* half-life and/or an increased serum half-life. The polypeptide part of the conjugate is often conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and an oligosaccharide moiety, to a lipophilic group and an oligosaccharide moiety, to an organic derivatizing agent and an oligosaccharide moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneously or sequentially. In a preferred embodiment of a polypeptide conjugated to different types of non-polypeptide moieties, the polypeptide is conjugated to one or more oligosaccharide moieties by *in vivo* glycosylation, and to one or more polymer molecules, preferably PEG, more preferably at an N-terminal, by conjugation *in vitro*.

Polypeptide of the invention

In a further aspect the invention relates to a modified FSH- α or a modified FSH- β polypeptide constituting part of a conjugate of the invention. The modified FSH- α and FSH- β are preferably glycosylated and thus further comprise N-linked and/or O-linked oligosaccharide moieties. Specific modified FSH- α and FSH- β polypeptides of the invention are those described in the section entitled "Conjugate of the invention".

Methods of preparing a conjugate of the invention

In the following sections "Conjugation to an oligosaccharide moiety", "Conjugation to a polymer molecule", "Conjugation to a lipophilic compound" and "Conjugation to an organic derivatizing agent", conjugation to specific types of non-polypeptide moieties is described.

Coupling to an oligosaccharide moiety

For *in vivo* glycosylation, conjugation to an oligosaccharide moiety takes place by means of a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic
5 plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, e.g. CHO K1, a BHK or HEK cell, e.g. HEK 293, an insect cell such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter. Preferred cells for expression of an *in vivo* glycosylated protein of the invention are mammalian cells, in particular CHO cells.

10

Conjugation to a polymer molecule

The polymer molecule to be coupled to the polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of 300-50,000 Da, such as 500-20,000 Da, more preferably
15 in the range of 1000-15,000 Da, such as in the range of 1000-12,000 Da or 2000-10,000 Da. Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH₂) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer which comprises different coupling groups, such as a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected
20 from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, polyvinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional
25 *in vivo* half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

PEG is the preferred polymer molecule, since it has only few reactive groups
30 capable of cross-linking compared to e.g. polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the

resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to control.

To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups. Suitable activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, AL, USA, or from PolyMASC Pharmaceuticals plc, UK. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and Pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference). Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG), BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in US 5,932,462 and US 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: US 5,824,778, US 5,476,653, WO 97/32607, EP 229,108, EP 402,378, US 4,902,502, US 5,281,698, US 5,122,614, US 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, US 5,736,625, WO 98/05363, EP 809 996, US 5,629,384, WO 96/41813, WO 96/07670, US 5,473,034, US 5,516,673, EP 605 963, US 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G.T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends

on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfhydryl, succinimidyl, maleimide, vinylsulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group (US 5,985,265). Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and where in the polypeptide such molecules are attached. The molecular weight of the polymer to be used will be chosen taking into consideration the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight and larger size (e.g. to reduce renal clearance), one may choose to conjugate either one or a few high molecular weight polymer molecules or a number of polymer molecules with a smaller molecular weight to obtain the desired effect. For epitope shielding, a sufficiently high number (e.g. 2-8, such as 3-6) of low molecular weight polymer molecules (e.g. with a molecular weight of about 5,000 Da) may be used to effectively shield all or most epitopes of the polypeptide.

When the protein is conjugated to only a single polymer molecule, for example where an N-terminal PEG molecule is the only polymer molecule, it will often be advantageous that the polymer molecule, which may be linear or branched, has a relatively high molecular weight, e.g. about 12-20 kDa.

In a specific embodiment, the polypeptide conjugate of the invention comprises a PEG molecule attached to most or substantially all of the lysine residues in the polypeptide available for PEGylation, in particular a linear or branched PEG molecule, e.g. with a molecular weight of about 5 kDa. In this case, it will normally be desirable to remove one or more of the lysines present in wildtype hFSH- α or hFSH- β in order to provide a more limited number of attachment sites and obtain a desired distribution of the PEG molecules. The polypeptide conjugate may further comprise a PEG molecule attached to the N-terminal amino acid residue in addition to the lysine residues.

Normally, the polymer conjugation is performed under conditions aiming at reacting as many of the available polymer attachment groups as possible with polymer mole-

cules. This is achieved by means of a suitable molar excess of the polymer in relation to the polypeptide. Typical molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1 or up to about 100-1. In some cases, the ratio may be somewhat lower, however, such as up to about 50-1, 10-1 or 5-1.

5 It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), *J. Biol. Chem.*, 252, 3578-3581; US 4,179,337; Shafer et al., (1986), *J. Polym. Sci. Polym. Chem.* 24, 375-378.

Subsequent to the conjugation residual activated polymer molecules are
10 blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules removed by a suitable method.

Covalent *in vitro* coupling of carbohydrate moieties glycosides (such as dextran) to amino acid residues of the polypeptide may also be used, e.g. as described in WO 87/05330 and in Aplin et al., *CRC Crit Rev. Biochem.*, pp. 259-306, 1981. The *in vitro* coupling of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can be
15 carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine groups to protein- and peptide-bound Gln residues in a so-called cross-linking reaction. The donor amine groups can be protein- or peptide-bound e.g. as the ϵ -amino group in Lys residues or can be part of a small or large organic molecule. An example of a small organic
20 molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., *Biochemistry* 35, 13072-13080).

TGases, in general, are highly specific enzymes, and not every Gln residue exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-
25 containing substances. On the contrary, only a few Gln residues function naturally as TGase substrates but the exact parameters governing which Gln residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino
30 acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2 -plasmin inhibitor, α -caseins, and β -caseins.

Conjugation to a lipophilic compound

The polypeptide and the lipophilic compound may be conjugated to each other either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamin, a carotenoid or steroid, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl, aryl, alkenyl or other multiple unsaturated compounds. The conjugation between the polypeptide and the lipophilic compound, optionally through a linker, may be done according to methods known in the art, e.g. as described by Bodanszky in *Peptide Synthesis*, John Wiley, New York, 1976 and in WO 96/12505.

Coupling to an organic derivatizing agent

Covalent modification of the polypeptide exhibiting FSH activity may be performed by reacting one or more attachment groups of the polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonate, pH 5.5-7.0, because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide is also useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction

be performed under alkaline conditions because of the high pKa of the guanidine functional group.

Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginy and glutaminy residues by reaction with ammonium ions.

10 *Blocking of a functional site*

It has been reported that excessive polymer conjugation can lead to a loss of activity of the polypeptide to which the polymer is conjugated. This problem can be eliminated by e.g. removal of attachment groups located at the functional site or by blocking the functional site prior to conjugation. The latter strategy constitutes a further embodiment of the invention (the first strategy being exemplified further above, e.g. by removal of lysine residues which may be located close to the functional site). More specifically, according to the second strategy the conjugation between the polypeptide and the non-polypeptide moiety is conducted under conditions where the functional site of the polypeptide is blocked by a helper molecule capable of binding to the functional site of the polypeptide.

Preferably, the helper molecule is one which specifically recognizes a functional site of the polypeptide, such as a receptor, in particular the FSH receptor or a part of the FSH receptor. Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the polypeptide exhibiting FSH activity. In particular, the helper molecule may be a neutralizing monoclonal antibody.

The polypeptide is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide is shielded or protected and consequently unavailable for derivatization by the non-polypeptide moiety such as a polymer. Following its elution from the helper molecule, the conjugate between the non-polypeptide moiety and the polypeptide can be recovered with at least a partially preserved functional site.

The subsequent conjugation of the polypeptide having a blocked functional site to a polymer, a lipophilic compound, an oligosaccharide moiety, an organic derivatizing agent

or any other compound is conducted in the normal way, e.g. as described in the sections above entitled "Conjugation to ...".

Irrespective of the nature of the helper molecule to be used to shield the functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free of or comprises only a few attachment groups for the non-polypeptide moiety of choice in any parts of the molecule where the conjugation to such groups will hamper the desorption of the conjugated polypeptide from the helper molecule. Hereby, selective conjugation to attachment groups present in non-shielded parts of the polypeptide can be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide moiety is a polymer molecule such as PEG which has the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially free of conjugatable epsilon amino groups, preferably free of any epsilon amino groups. Accordingly, in a preferred embodiment the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free of any conjugatable attachment groups for the non-polypeptide moiety of choice.

In a further embodiment the helper molecule is first covalently linked to a solid phase such as column packing materials, for instance Sephadex or agarose beads, or a surface, e.g. a reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying the helper molecule and conjugation carried out according to methods known in the art, e.g. as described in the sections above entitled "Conjugation to ...". This procedure allows the polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide conjugate is eluted by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule may be derivatised with a second molecule (e.g. biotin) that can be recognized by a specific binder (e.g. streptavidin). The specific binder may be linked to a solid phase thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate may be released from the helper molecule in any appropriate fashion. Deprotection may be achieved by providing conditions in

which the helper molecule dissociates from the functional site of the FSH to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH.

5 *Conjugation of a tagged polypeptide*

In an alternative embodiment the polypeptide is expressed as a fusion protein with a tag, i.e. an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide and the non-polypeptide moiety. In particular, the tag may be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide can be immobilised via the tag. The conjugation to the tagged polypeptide in, e.g., microtiter plates has the advantage that the tagged polypeptide can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag may function as a spacer molecule ensuring an improved accessibility to the immobilised polypeptide to be conjugated. The conjugation using a tagged polypeptide may be to any of the non-polypeptide moieties disclosed herein, e.g. to a polymer molecule such as PEG.

The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, e.g. from Unizyme Laboratories, Denmark. For instance, the tag may consist of any of the following sequences:

His-His-His-His-His-His
Met-Lys-His-His-His-His-His
Met-Lys-His-His-Ala-His-His-Gln-His-His
Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln
Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-Gln

or any of the following:

EQKLI SEEDL (a C-terminal tag described in *Mol. Cell. Biol.* 5:3610-16, 1985)

DYKDDDDK (a C- or N-terminal tag)

YPYDVPDYA

Antibodies against the above tags are commercially available, e.g. from ADI, Aves Lab and Research Diagnostics.

The subsequent cleavage of the tag from the polypeptide may be achieved by
5 use of commercially available enzymes.

Methods for preparing a polypeptide of the invention or the polypeptide of the conjugate of the invention

The polypeptide of the present invention or the polypeptide part of a conjugate
10 of the invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. Polypeptides of the invention may also be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

15 FSH- α and FSH- β are preferably expressed by the same host cell, thus becoming dimerized *in vivo* prior to purification and possible *in vitro* conjugation to a non-polypeptide moiety. Co-expression of FSH- α and FSH- β in CHO cells is e.g. described by Keene et al., *J Biol Chem* 1989 25; 264(9): 4769-75. Alternatively, the polypeptide may be expressed as a single-chain polypeptide wherein the nucleotide sequences encoding FSH- α
20 and FSH- β are fused, either directly or using a suitable peptide linker, and expressed as a single-chain polypeptide using a similar approach to that described in US 5,883,073 or WO 96/05224. It will thus be understood that the polypeptide of the invention may comprise the FSH- α and FSH- β subunits in the form of two separate polypeptide chains, where the two chains become dimerized *in vivo* so as to form a dimeric polypeptide, or it may comprise a
25 single-chain construct comprising the two subunits covalently linked by a peptide bond or a peptide linker.

In an alternative embodiment, two FSH- β subunits, wherein at least one of the two β subunits is modified as described herein, preferably by introduction of at least one N- or O-glycosylation site, may be expressed as a single-chain polypeptide in which the subunits are
30 either fused directly or via a peptide linker. Similarly, two FSH- α subunits, wherein at least one of the two α subunits is modified as described herein, may also be expressed as a single-chain polypeptide with the subunits fused directly or via a peptide linker. Further, it is also

possible to produce single-chain constructs comprising more than two subunits, e.g. three subunits, wherein at least one of the individual subunits is modified as described herein, and wherein the subunits are fused to each other directly or via a peptide linker. For example, a single-chain construct having the sequence FSH α -FSH β -FSH β , FSH β -FSH α -FSH β or FSH β -FSH β -FSH α , wherein the β subunits in each construct are the identical or different, may be produced using techniques known in the art. Single-chain constructs of this general type are disclosed in US 5,705,478, US 5,883,073, WO 99/25489 and WO 96/05224.

For single-chain constructs, the linker peptide will often predominantly include the amino acid residues Gly, Ser, Ala and/or Thr. Such a linker typically comprises 1-30 amino acid residues, such as a sequence of about 2-20 or 3-15 amino acid residues. The amino acid residues selected for inclusion in the linker peptide should exhibit properties that do not interfere significantly with the activity of the polypeptide. Thus, the linker peptide should on the whole not exhibit a charge which would be inconsistent with the desired FSH activity, or interfere with internal folding, or form bonds or other interactions with amino acid residues in one or more of the subunits which would seriously impede the binding of the dimeric or multimeric polypeptide to the receptor.

Specific linkers for use in the present invention may be designed on the basis of known naturally occurring as well as artificial polypeptide linkers (see, e.g., Hallewell et al. (1989), *J. Biol. Chem.* 264, 5260-5268; Alfthan et al. (1995), *Protein Eng.* 8, 725-731; Robinson & Sauer (1996), *Biochemistry* 35, 109-116; Khandekar et al. (1997), *J. Biol. Chem.* 272, 32190-32197; Fares et al. (1998), *Endocrinology* 139, 2459-2464; Smallshaw et al. (1999), *Protein Eng.* 12, 623-630; US 5,856,456). For instance, linkers used for creating single-chain antibodies, e.g. a 15mer consisting of three repeats of a Gly-Gly-Gly-Gly-Ser amino acid sequence ((Gly₄Ser)₃), are contemplated to be useful. Furthermore, phage display technology as well as selective infective phage technology can be used to diversify and select appropriate linker sequences (Tang et al., *J. Biol. Chem.* 271, 15682-15686, 1996; Hennecke et al. (1998), *Protein Eng.* 11, 405-410). Also, Arc repressor phage display has been used to optimize the linker length and composition for increased stability of a single-chain protein (Robinson and Sauer (1998), *Proc. Natl. Acad. Sci. USA* 95, 5929-5934). Another way of obtaining a suitable linker is by optimizing a simple linker, e.g. ((Gly₄Ser)_n), through random mutagenesis. The linker may e.g. be (Gly₄Ser)_n or (Gly₃Ser)_n where n is 1, 2, 3 or 4.

The nucleotide sequence encoding FSH- α or FSH- β modified according to the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the

parent FSH subunit, such as hFSH- α or hFSH- β with the amino acid sequence shown in SEQ ID NO:2 or 4, respectively, or the precursor form thereof (shown in SEQ ID NO:1 and 3, respectively) and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or deletion (i.e. removal or substitution) of the relevant amino acid residue(s). The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence may be prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, *PNAS* 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FSH in the desired transformed host cell.

It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding a polypeptide described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

The recombinant vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for mammalian eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jolla, CA, USA). Useful expression vectors for yeast cells include the 2 μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, *Ann. New York Acad. Sci.* 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., *Cell* 45, pp. 685-98 (1986)), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, *Mol. Cell. Biol.* 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., US 5,122,464 and EP 338,841).

In one embodiment, a pair of expression vectors are used for expressing the polypeptide subunits of the invention. Each of the vectors of said pair is capable of transfecting a eukaryotic cell as described herein, and the vectors comprise nucleotide sequences encoding, respectively, a modified FSH- α as described herein and a wildtype FSH- β subunit, a modified FSH- β as described herein and a wildtype FSH- α subunit, or a modified FSH- α and

a modified FSH- β as described herein. The use of a pair of vectors is e.g. described in EP 211,894. Alternatively, a single expression vector comprising nucleotide sequences encoding both the FSH- α subunit and the FSH- β subunit, where at least one of the subunits is modified as described herein, may be used for expressing the polypeptide subunits.

5 The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication.

10 The vector may also comprise a selectable marker, e.g. a gene whose product complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, *Gene* 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces*
15 *cerevisiae*, selectable markers include *ura3* and *leu2*. For filamentous fungi, selectable markers include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

 The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the
20 polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

 A wide variety of expression control sequences may be used in the present in-
25 vention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

 Examples of suitable control sequences for directing transcription in mammal-
30 ian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1 α (EF-1 α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) pro-

moter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α -mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α -amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the *lac* system, the *trp* system, the *TAC* or *TRC* system, and the major promoter regions of phage lambda.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α -amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., *Protein Expression and Purification* 4,

349-357 (1993) or human pancreatic lipase (hpl) (*Methods in Enzymology* 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFSH or the murine Ig kappa light chain signal peptide (Coloma, M (1992) *J. Imm. Methods* 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α -factor signal peptide from *S. cerevisiae* (cf. US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., *Cell* 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., *Yeast* 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide *ompA* (EP581821).

The nucleotide sequences of the invention encoding the dimeric polypeptide exhibiting FSH activity, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may optionally also include a nucleotide sequence that encodes a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide may be homologous (e.g. be that normally associated with a hFSH subunit) or heterologous (i.e. originating from another source than hFSH) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

Any suitable host may be used to produce the polypeptide subunits of the invention, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include gram-positive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis*, or *Streptomyces*, or gram-negative bacteria, such as *Pseudomonas* or strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A.*

oryzae, *A. niger*, or *A. nidulans*, *Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for

5 transforming *Fusarium* species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors,

10 *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *PNAS USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptera* cell line, such as

15 *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusia ni* cells (High Five) (US 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g.

20 NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, USA. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation,

25 DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel et al. (eds.), 1996, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in

30 (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, NJ, USA and Harrison MA and Rae IF, *General Techniques of Cell Culture*, Cambridge University Press 1997).

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g. in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, it can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The resulting polypeptide may be recovered by methods known in the art. For example, it may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g. ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g. preparative isoelectric focusing), differential solubility (e.g. ammonium sulfate precipitation), SDS-PAGE, or extraction (see e.g. *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Pharmaceutical composition of the invention and its use

In one aspect the polypeptide, the conjugate or the pharmaceutical composition according to the invention is used for the manufacture of a medicament for treatment of infertility or diseases associated with insufficient endogenous production of FSH.

In another aspect the polypeptide, the conjugate or the pharmaceutical composition according to the invention is used in a method of treating an infertile mammal, in particular a human, comprising administering to the mammal in need thereof such polypeptide, conjugate or pharmaceutical composition.

The polypeptide exhibiting FSH activity of the invention or the conjugate of the invention is administered at a dose approximately paralleling that employed in therapy with rhFSH such as Gonal-F® and Puregon®. However, due to the increased functional *in*

in vivo half-life of the conjugate of the invention, it is contemplated that the product will be administered less frequently and at a dose which provides a comparable effect to that obtained in current therapy. It is thus contemplated that the composition of the invention may be administered at substantially less frequent intervals than currently available treatments, e.g. not more often than once every three days, such as not more than once every four, five, six or seven days. Accordingly, the exact dose to be administered will depend on the circumstances, including the patient to be treated, the cause of infertility if known, the status of the ovaries, the patient's plasma FSH concentration prior to treatment, and the functional *in vivo* half-life of the product. Normally, in the treatment of infertility the dose should be capable of stimulating follicle maturation, e.g. induce follicles to grow about 2 mm per day during a time period of 8-9 days. For instance, for a product having a functional *in vivo* half-life of 3-4 days, two doses should be given at least three days apart if a relatively stable plasma concentration is desired. Analogously, for a product having a functional *in vivo* half-life of about 6 days, one dose would suffice during most of the stimulation period.

The composition of the invention may be exceedingly advantageous when employed in a step-down protocol, i.e. a protocol where decreasing dosages of FSH are given during the stimulation period, but where use of the composition of the invention, e.g. administered in one or two doses as outlined above, may provide such a slowly decreasing plasma concentration of FSH.

It will be apparent to those of skill in the art that an effective amount of a conjugate, preparation or composition of the invention depends, *inter alia*, upon the disease, the dose, the administration schedule, whether the polypeptide or conjugate or composition is administered alone or in conjunction with other therapeutic agents, the serum half-life of the compositions, and the general health of the patient. Typically, an effective dose of the conjugate, preparation or composition of the invention is sufficient to ensure development and maturation of follicles at a rate and to a degree compatible with that obtained using standard rhFSH such as Gonal-F® and Puregon®.

A further contemplated advantage is that the more stable plasma concentration obtained with a composition of the invention results in a more efficient development and maturation of follicles, which subsequently may enable a higher pregnancy rate.

The polypeptide or conjugate of the invention is normally administered in a composition including one or more pharmaceutically acceptable carriers or excipients. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects

in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art, and the polypeptide or conjugate of the invention can be formulated into pharmaceutical compositions by well-known methods (see e.g. Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company
5 (1990); Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis (2000); and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press (2000)). Pharmaceutically acceptable excipients that may be used in compositions comprising the polypeptide or conjugate of the invention include, for example, buffering agents, stabilizing agents, preservatives, isotonicifiers, non-
10 ionic surfactants or detergents ("wetting agents"), antioxidants, bulking agents or fillers, chelating agents and cosolvents.

The pharmaceutical composition of the polypeptide or conjugate of the invention may be formulated in a variety of forms, including liquids, e.g. ready-to-use solutions or suspensions, gels, lyophilized, or any other suitable form, e.g. powder or crystals suitable for
15 preparing a solution. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

The pharmaceutical composition containing the polypeptide or conjugate of the invention may be administered intravenously, intramuscularly, intraperitoneally, intradermally, subcutaneously, sublingually, buccally, intranasally, transdermally, by inhalation, or in
20 any other acceptable manner, e.g. using PowderJect® or ProLease® technology or a pen injection system. The preferred mode of administration will depend upon the particular indication being treated and will be apparent to one of skill in the art. In particular, it is advantageous that the composition be administered subcutaneously, since this allows the patient to conduct the administration herself.

25 The pharmaceutical composition of the invention may be administered in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide or conjugate of the invention, either concurrently or in accordance with any other acceptable treatment schedule. In addition, the polypeptide, conjugate or pharmaceutical composition of the
30 invention may be used as an adjunct to other therapies.

By obtaining a more stable FSH plasma concentration just above the threshold level for follicle growth, the composition of the invention is of particular interest for the treat-

treatment of women suffering from anovulation WHO type I, II or III, since only 1-2 mature follicles are desired in these patients.

Furthermore, the invention relates in other aspects to the use of a composition of the invention in a step-down protocol where a decreasing plasma FSH concentration is obtained using only one or two injections, and preferably only a single injection, to the use of a composition of the invention in a step-up protocol where an increase in FSH concentrations is obtained faster using a lower individual as well as total dosage, and to the use of a composition of the invention in combination with compounds for *in vitro* maturation (sterol derivatives such as FF-MAS and media containing growth and maturation factors known in the art).

Mixtures of FSH and LH activities (hMG) are routinely used in the treatment of human infertility. This particular combination therapy may be advantageous because gonadal support of gamete maturation is dependent upon the synergistic actions of both FSH and LH. Current treatment protocols requiring FSH and LH activity utilize urinary extracts from postmenopausal women. The use of these extracts is compromised by several factors, including variability.

It will in some cases be advantageous to administer the composition of the invention as part of a treatment protocol that also involves LH and/or hCG, for example recombinant LH and/or hCG. This may in particular be useful for treatment of women with low endogenous LH levels. Finally, the composition of the invention may be used, possibly in combination with LH, in the treatment of male infertility, in particular of hypogonadotropic hypogonadism and oligo- or azoospermia. The more stable plasma concentration obtained with a composition of the invention may lead to a more efficient spermatogenesis. Also, a long lasting effect would be particularly advantageous for such treatment due to the long-term treatment period of about three months.

The present invention will be further illustrated by the following non-limiting methods and examples.

Structure analysis methods

Sequence numbering

The amino acid sequence of hFSH- α is numbered according to the mature sequence shown in SEQ ID NO:2; an (a) suffix herein indicates the α chain. The amino acid

sequence of hFSH- β is numbered according to the mature sequence shown in SEQ ID NO:4; a (b) suffix herein indicates the β chain.

Structures

5 Human FSH- α is identical to the α chain of Human Chorionic Gonadotropin (HCG) for which two published structures are available: Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., Hendrickson, W. A.: *Structure* 2 pp. 545 (1994) and Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., Isaacs, N. W.: *Nature* 369 pp. 455 (1994), both including the β chain of HCG. The β chain of hFSH is
10 32 percent identical to the amino acid sequence of the structural part of the β chain of HCG (see the sequence alignment of Figure 1). A series of 50 models of the 3D structure of FSH was built based on the above two available hCG structures and based on the sequence alignment in Figure 1 using the program Modeller 98 (MSI Inc., 1999). The four N-terminal residues (A1(a), P2(a), D3(a) and V4(a) as well as the three C-terminal residues (H90(a), K91(a)
15 and S92(a) were not modeled as they are not identified in the HCG structures. All of the hFSH- β chain was modeled, even the part which has no homologous residues in the HCG structures.

Accessible Surface Area (ASA)

20 The computer program Access (B. Lee and F.M. Richards, *J. Mol. Biol.* 55: 379-400 (1971)) version 2 (©1983 Yale University) was used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be
25 removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of side chain

The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain
30 atoms of that residue type in an extended Ala-x-Ala tripeptide, see Hubbard, Campbell & Thornton (1991) *J. Mol. Biol.* 220,507-530. For this example the CA atom is regarded as be-

ing a part of the side chain of glycine residues but not other residues. The following values are used as standard 100% ASA for the side chain:

Ala	69.23	Å ²	Leu	140.76	Å ²
Arg	200.35	Å ²	Lys	162.50	Å ²
Asn	106.25	Å ²	Met	156.08	Å ² ⁵
Asp	102.06	Å ²	Phe	163.90	Å ²
Cys	96.69	Å ²	Pro	119.65	Å ²
Gln	140.58	Å ²	Ser	78.16	Å ²
Glu	134.61	Å ²	Thr	101.67	Å ² ¹⁰
Gly	32.28	Å ²	Trp	210.89	Å ²
His	147.00	Å ²	Tyr	176.61	Å ²
Ile	137.91	Å ²	Val	114.14	Å ²

15

Determination of surface exposed residues from structural models:

Surface accessibility and fractional ASA of side chains were calculated for each of the 50 model structures. The average value over the structural ensemble was used in the following. The N- and C-terminal residues of the FSH- α chain not included in the model are defined as having 100% side chain accessibility.

The following amino acid residues in hFSH- α and hFSH- β , respectively, have more than 25% of their side chain exposed to the surface:

A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), L12(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), P24(a), L26(a), M29(a), F33(a), R42(a), S43(a), K44(a), K45(a), T46(a), L48(a), V49(a), Q50(a), N52(a), V61(a), K63(a), S64(a), Y65(a), N66(a), R67(a), V68(a), T69(a), M71(a), G72(a), G73(a), F74(a), K75(a), N78(a), T80(a), A81(a), H83(a), C84(a), S85(a), T86(a), Y88(a), Y89(a), H90(a), K91(a), S92(a), N1(b), S2(b), E4(b), L5(b), T6(b), N7(b), I8(b), T9(b), K14(b), E15(b), E16(b), R18(b), F19(b), I21(b), S22(b), N24(b), Y31(b), Y33(b), R35(b), D36(b), L37(b), Y39(b), K40(b), D41(b), P42(b), A43(b), R44(b), P45(b), K46(b), I47(b), K49(b), K54(b), E55(b), L56(b), V57(b), Y58(b), E59(b), T60(b), V61(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), Y74(b), T75(b), T80(b), Q81(b), H83(b), G85(b), K86(b), D88(b), S89(b), D90(b), S91(b), D93(b), T95(b), V96(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), K110(b), and E111(b).

35

The following amino acid residues have more than 50% of their side chain exposed to the surface:

A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), K45(a), T46(a), L48(a), V49(a), Q50(a), N52(a), K63(a), S64(a), N66(a), R67(a), T69(a), G72(a), G73(a), K75(a), T86(a), Y89(a), H90(a), K91(a), S92(a), N1(b), N7(b), T9(b), E15(b), E16(b), R18(b), F19(b), N24(b),
 5 Y33(b), D41(b), P42(b), A43(b), R44(b), P45(b), K46(b), I47(b), K54(b), E55(b), V57(b), Y58(b), E59(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), T75(b), Q81(b), H83(b), K86(b), D88(b), S89(b), D90(b), S91(b), T95(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), K110(b), and E111(b).

10 Determining distances between atoms

The distance between atoms is most easily determined using molecular graphics software, e.g. InsightII v. 98.0, MSI Inc.

Example 1

15 Construction of plasmids for expression of FSH

A gene encoding the human FSH- α subunit was constructed by assembly of synthetic oligonucleotides by PCR using methods similar to the ones described in Stemmer et al. (1995) *Gene* 164, pp. 49-53. The native FSH- α signal sequence was maintained in order to allow secretion of the gene product. The codon usage of the gene was optimised for high ex-
 20 pression in mammalian cells. Furthermore, in order to achieve high gene expression, an intron (from pCI-Neo (Promega)) was included in the 5' untranslated region of the gene. The synthetic gene was subcloned behind the CMV promoter in pcDNA3.1/Hygro (Invitrogen). The sequence of the resulting plasmid, termed pBvdH977, is given in SEQ ID NO:5 (FSH- α -coding sequence at position 1225 to 1570). Similarly, a synthetic gene encoding the wildtype
 25 human FSH- β subunit was constructed. Also in this construct, the native signal sequence was maintained (except for a Lys to Glu mutation at position 2) in order to allow secretion, and the codon usage was optimised for high expression and an intron was included in the recipient vector (pcDNA3.1/Zeo (Invitrogen)). The sequence of the resulting FSH- β -containing plasmid, termed pBvdH1022, is given in SEQ ID NO:6 (FSH- β -coding sequence at position 1231
 30 to 1617). A plasmid containing both the FSH- α and the FSH- β encoding synthetic genes was generated by subcloning the FSH- α containing *NruI*-*PvuII* fragment from pBvdH977 into

pBvdH1022 linearized with *NruI*. The resulting plasmid, in which the FSH- α and FSH- β -expression cassettes are in direct orientation, was termed pBvdH1100.

Example 2

5 Expression of FSH in CHO cells

FSH was expressed in Chinese Hamster Ovary (CHO) K1 cells, obtained from the American Type Culture Collection (ATCC, CCL-61).

For transient expression of FSH, cells were grown to 95% confluency in serum-containing media (MEM α with ribonucleotides and deoxyribonucleotides (Life Technologies Cat # 32571-028) containing 1:10 FBS (BioWhittaker Cat # 02-701F) and 1:100 penicillin and streptomycin (BioWhittaker Cat # 17-602E), or Dulbecco's MEM/Nut.-mix F-12 (Ham) L-glutamine, 15 mM Hepes, pyridoxine-HCl (Life Technologies Cat # 31330-038) with the same additives. FSH-encoding plasmids were transfected into the cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's specifications.

15 24-48 hrs after transfection, culture media were collected, centrifuged and filtered through 0.22 μ m filters to remove cells.

Stable clones expressing FSH were generated by transfection of CHO K1 cells with FSH-encoding plasmids followed by incubation of the cells in selective media (for instance one of the above media containing 0.5 mg/ml zeocin for cells transfected with plasmid pBvdH1100). Stably transfected cells were isolated and sub-cloned by limited dilution.

20 Clones that produced high levels of FSH were identified by ELISA (see below).

Example 3

Large-scale production of FSH in CHO cells

25 The cell line CHO K1 1100-5, stably expressing human FSH, was passed 1:10 from a confluent culture and propagated as adherent cells in serum-containing medium Dulbecco's MEM/Nut.-mix F-12 (Ham) L-glutamine, 15 mM Hepes, pyridoxine-HCl (Life Technologies Cat # 31330-038), 1:10 FBS (BioWhittaker Cat # 02-701F), 1:100 penicillin and streptomycin (BioWhittaker Cat # 17-602E) until confluence in a 10 layer cell factory (NUNC #165250). The media was then changed to serum-free media: Dulbecco's MEM/Nut.-mix F-12 (Ham) L-glutamine, pyridoxine-HCl (Life Technologies Cat # 21041-025) with the addition of 1:500 ITS-A (Gibco/BRL # 51300-044), 1:500 EX-CYTE VLE (Serological Proteins

30

Inc. # 81-129) and 1:100 penicillin and streptomycin (BioWhittaker Cat # 17-602E). Subsequently, every 24 h, culture media were collected and replaced with 1 fresh liter of the same serum-free media. The collected media was filtered through 0.22 μ m filters to remove cells. Growth in cell factories was continued with daily harvests and replacements of the culture media until FSH yields dropped below one-fourth of the initial expression level (typically after 10-15 days).

Example 4

Analysis of FSH forms by Western blotting and isoelectric focusing

The FSH content of samples was analysed by Western blotting: Proteins were separated by SDS-PAGE and a standard Western blot was performed using rabbit anti human FSH (AHP519, Serotec) or mouse anti human FSH- β (MCA338, Serotec) as primary antibody, and an ImmunoPure Ultra Sensitive ABC Peroxidase Staining Kit (Pierce) for detection. Wild-type FSH produced as described above in Examples 1-3 was found to have the same mobility as FSH from references such as Puregon® (Organon) or Gonal-F® (Serono).

For analysis of pI, samples were separated on pH 3-7 IEF gels (NOVEX). After electrophoresis, proteins were blotted onto Immobilon-P (Millipore) membranes and a Western blot was performed as described above, using the same antibodies and detection kit. In accordance with published observations (see, for instance, Loumaye et al. (1998) *Human Reprod. Update* 4, 862-881), various FSH isoforms were detected, mostly in the pH 4-5.2 range for wildtype FSH. This is due to heterogeneity in carbohydrate content, most importantly sialic acid.

Example 5

Purification of FSH wildtype and variants

Three chromatographic steps were employed to obtain highly purified FSH. First an anion exchanger step, then hydrophobic interaction chromatography (HIC) and finally an immunoaffinity step using an FSH- β specific monoclonal antibody.

Culture supernatants were prepared as described in Example 3. Filtered culture supernatants were concentrated 10 to 20 times by ultrafiltration (10 kD cut-off membrane), pH was adjusted to 8.0 and conductivity to 10 - 15 mS/cm, before application on a DEAE Sepharose (Pharmacia) anion exchanger column, which had been equilibrated in ammonium

acetate buffer (0.16 M, pH 8.0). Semipurified FSH was recovered both in the unbound flow-through fraction as well as in the wash fraction using 0.16 M ammonium acetate, pH 8.0. The flow through and wash fractions were pooled and ammonium sulfate was added from a stock solution (4.5 M) to obtain a final concentration of 1.5 M $(\text{NH}_4)_2\text{SO}_4$. The pH was adjusted to 7.0.

The partially purified FSH was subsequently applied on a 25 ml butyl Sepharose (Pharmacia) HIC column. After application, the column was washed with at least 3 column volumes of 1.5 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM ammonium acetate, pH 7 (until the absorbance at 280 nm reached baseline level) and FSH was eluted with 4 column volumes of buffer B (20 mM ammonium acetate, pH 7). FSH enriched fractions from the HIC step were pooled, concentrated and diafiltrated using Vivaspin 20 modules, 10 kD cut-off membrane (Vivascience), to a 50 mM sodium phosphate, 150 mM NaCl, pH 7.2.

For the third chromatographic step, an anti-FSH- β monoclonal antibody (RDI-FSH909, Research Diagnostics) was immobilized to CNBr-activated Sepharose (Pharmacia) using a standard procedure from the supplier. Approximately 1 mg antibody was coupled per ml resin. The immunoaffinity resin was packed in plastic columns and equilibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.2 before application.

The buffer exchanged eluate from the butyl HIC step was applied on the antibody column by use of gravity flow. This was followed by several washing steps in 50 mM sodium phosphate solutions (0.5 M NaCl and 1 M NaCl, both pH 7.2). Elution was performed using either 1 M NH_3 or 0.6 M NH_3 , 40% (v/v) isopropanol and the eluate was immediately neutralized with 1 M acetic acid to pH 6-8.

The purified FSH bulk product was concentrated and diafiltrated using Vivaspin 20 modules, 10 kD cut-off membrane (Vivascience), to a 50 mM sodium phosphate, 150 mM NaCl, pH 7.2. For subsequent storage, BSA was added to 0.1% (w/v) and the purified FSH was microfiltrated using a 0.22 μm filter prior to storage at -80°C .

SDS-PAGE, run under non-dissociating conditions (without boiling), showed wildtype FSH migrating as an apparent 42 ± 3 kDa band, slightly diffuse due to heterogeneity in the attached carbohydrates. The purity was about 80-90%. N-terminal sequencing showed that the α -chain had the expected N-terminal sequence starting with residue 1 (SEQ ID NO:2) and the β -chain starting with residue 3 (SEQ ID NO:4). These N-terminal sequences have

been found previously for recombinant FSH produced in CHO cells (Olijve, W. et al. (1996) *Mol. Hum. Reprod.* 2, 371-382).

Example 6

5 FSH in vitro activity assay

6.1 FSH assay Outline

It has previously been published that activation of the FSH receptor by FSH leads to an increase in the intracellular concentration of cAMP. Consequently, transcription is activated at promoters containing multiple copies of the cAMP response element (CRE). It is
10 thus possible to measure FSH activity by use of a CRE luciferase reporter gene introduced into CHO cells expressing the FSH receptor.

6.2 Construction of a CHO FSH-R / CRE-luc cell line

Stable clones expressing the human FSH receptor were produced by transfection of CHO K1 cells with a plasmid containing the receptor cDNA inserted into pcDNA3
15 (Invitrogen) followed by selection in media containing 600 µg/ml G418. Using a commercial cAMP-SPA RIA (Amersham), clones were screened for the ability to respond to FSH stimulation. On the basis of these results, an FSH receptor-expressing CHO clone was selected for further transfection with a CRE-luc reporter gene. A plasmid containing the reporter gene
20 with 6 CRE elements in front of the Firefly luciferase gene was co-transfected with a plasmid conferring Hygromycin B resistance. Stable clones were selected in the presence of 600 µg/ml G418 and 400 µg/ml Hygromycin B. A clone yielding a robust luciferase signal upon stimulation with FSH ($EC_{50} \sim 0.01$ IU/ml) was obtained. This CHO FSH-R / CRE-luc cell line was used to measure the activity of samples containing FSH.

25

6.3 FSH luciferase assay

To perform activity assays, CHO FSH-R / CRE-luc cells were seeded in white
96 well culture plates at a density of about 15,000 cells/well. The cells were in 100 µl
DMEM/F-12 (without phenol red) with 1.25% FBS. After incubation overnight (at 37°C, 5%
30 CO₂), 25 µl of sample or standard diluted in DMEM/F-12 (without phenol red) with 10% FBS was added to each well. The plates were further incubated for 3 hrs, followed by addition

of 125 μ l LucLite substrate (Packard Bioscience). Subsequently, plates were sealed and luminescence was measured on a TopCount luminometer (Packard) in SPC (single photon counting) mode.

5 **Example 7**

FSH ELISA

The concentration of FSH in samples was quantified by use of a commercial immunoassay (DRG FSH EIA, DRG Instruments GmbH, Marburg, Germany). DRG FSH EIA is a solid phase immunosorbent assay (ELISA) based on the sandwich principle. The
10 microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the FSH- β subunit. An aliquot of FSH-containing sample (diluted in H₂O with 0.1% BSA) and an anti-FSH antiserum conjugated with horseradish peroxidase are added to the coated wells. After incubation, unbound conjugate is washed off with water. The amount of bound peroxidase is proportional to the concentration of FSH in the sample. The intensity of
15 colour developed upon addition of substrate solution is proportional to the concentration of FSH in the sample.

Example 8

Animal studies

20 The pharmacokinetic profile of FSH and variant forms was determined as follows: Immature 26-27 days old female Sprague-Dawley rats were injected i.v. with 3-4 μ g FSH, produced, purified and analyzed as described in Examples 1-7. Subsequently, blood samples were taken at various time-points after injection. FSH concentrations in serum samples were determined by ELISA, as described in Example 7.

25 *In vivo* bioactivity of wildtype recombinant FSH and variant forms may be evaluated by the ovarian weight augmentation assay (Steelman and Pohley (1953) *Endocrinology* **53**, 604-616). Furthermore, the ability of FSH and variant forms to stimulate maturation of follicles in laboratory animals may be detected with e.g. ultrasound equipment.

Example 9Construction and analysis of a variant form of FSH containing two N-linked glycosylations at the N-terminus of the α subunit

A construct encoding a modified form of FSH- α , having two additional sites
5 for N-linked glycosylation at its N-terminus was generated by site-directed mutagenesis using
standard DNA techniques known in the art. A DNA fragment encoding the sequence Ala-
Asn-Ile-Thr-Val-Asn-Ile-Thr-Val was inserted immediately upstream of the mature FSH- α
sequence in pBvdH977. The sequence of the resulting plasmid, termed pBvdH1163, is given
in SEQ ID NO:7 (modified FSH- α -encoding sequence at position 1225 to 1599). A plasmid
10 encoding both subunits was constructed by subcloning the FSH-containing *NruI-PvuII* frag-
ment from pBvdH1163 into pBvdH1022 (Example 1), which had been linearized with *PvuII*.
The resulting plasmid was termed pBvdH1208.

For expression of the variant form of FSH containing two N-linked glycosyla-
tions at the N-terminus of the α subunit (termed FSH1208), CHO K1 cells were transfected
15 with pBvdH1208 or co-transfected with a combination of pBvdH1163, encoding the modified
 α subunit and pBvdH1022, encoding the wildtype β subunit. Transient expressions, isolation
of stable expression clones, and large-scale production of FSH1208 were performed as de-
scribed for wildtype FSH in Examples 2 and 3.

Western blotting and isoelectric focusing were performed as described in Ex-
20 ample 4. Western blotting showed that FSH1208 had a larger molecular mass than wildtype
FSH, indicating that the introduction of acceptor sites for N-linked glycosylation at the N-
terminus of the α subunit indeed lead to hyperglycosylation of FSH. Isoelectric focusing
demonstrated that the FSH forms in the FSH1208 samples were found in a lower pI range
than wildtype FSH. Thus, the pH interval for FSH1208 isoforms was about 3.0-4.5 versus
25 about 4.0-5.2 for wildtype FSH. This indicated that FSH1208 molecules are on average more
negatively charged than the wild type, which is attributed to the presence of additional sialic
acid residues.

FSH1208 was purified and characterized as described in Example 5. SDS-
PAGE, run under non-dissociating conditions (without boiling), showed FSH1208 migrating
30 as an apparent 55 ± 5 kDa band, slightly diffuse due to heterogeneity in the attached carbohy-
drates. The purity was about 80-90%. N-terminal sequencing showed that while the β -chain
had the same N-terminal sequence as wildtype FSH, the sequence of α -chain was in agree-

ment with this subunit carrying the expected N-terminal extension ANITVNTTV, in which both asparagines residues are glycosylated.

The specific activity of FSH1208 was determined by measurement of the *in vitro* bioactivity (FSH luciferase assay, Example 6) and the FSH content of the samples (FSH ELISA, Example 7). The specific activity of FSH1208 was found to be about one-third of that of the wildtype reference.

A pharmacokinetic study performed as described in Example 8 showed that 24 hours after injection of equal amounts of wildtype FSH and FSH1208, the sera of FSH1208-treated animals contained more than 10 fold more remaining immunoreactive material than the sera from animals treated with wildtype FSH.

Example 10

Construction and analysis of other FSH variants containing additional glycosylation sites

Plasmids encoding variant forms of FSH- α and FSH- β containing additional sites for N-linked glycosylation were generated by site-directed mutagenesis using standard DNA techniques known in the art. The following amino acid substitutions and/or insertions were generated:

FSH1147: Amino acid Tyr58 of mature FSH- β altered to Asn

FSH1349: N-terminus of mature FSH- α altered from APD QDC... to:

APNDTVNFT QDC ...

FSH1354: N-terminus of mature FSH- β altered from NS CEL ... to:

NSNITVNTTV CEL ...

Plasmids encoding the variant forms were transiently expressed in CHO K1 cells as described in Example 2. Plasmids encoding FSH- α variants were co-transfected with a plasmid encoding wild-type FSH- β and vice versa.

Western and isoelectric focusing were performed on culture media samples as described in Example 4. The variant forms had higher molecular weights than the wild-type, indicating that the additional acceptor sites for N-linked glycosylation had indeed been glycosylated. Furthermore, isoelectric focusing showed that the different isoforms of the three FSH variants were spread over a lower pI range than the wildtype. This strongly suggests that the variant forms had a higher sialic acid content than the wildtype.

In vitro FSH activities of the resulting media samples were analysed as described in Example 6.3. All three variant forms were able to stimulate the CHO FSH-R / CRE-luc cells, indicating that these variant FSH forms have retained significant FSH activity.

5 While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above may be used in various combinations. All publications, patents,
10 patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

CLAIMS

1. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising
 - i) a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein at least one of said FSH- α and FSH- β subunits differs from the corresponding wild-
5 type subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed, and
 - ii) at least one non-polypeptide moiety bound to an attachment group of at least one of said subunits.
- 10 2. The conjugate of claim 1, wherein the amino acid sequence of at least one of said FSH- α and FSH- β subunits differs from that of the corresponding wildtype subunit in that an amino acid residue comprising an attachment group for the non-polypeptide moiety has been introduced.
- 15 3. The conjugate of claim 2, wherein the introduced attachment group is selected from the group consisting of an N-glycosylation site, an O-glycosylation site, and an attachment group for a polymer molecule, a lipophilic compound, a carbohydrate moiety or an organic derivatizing agent.
- 20 4. The conjugate of any of claims 1-3, comprising at least one PEG molecule attached to an attachment group of at least one of the subunits.
5. The conjugate of any of claims 1-4, comprising at least one introduced N-glycosylation site, and further comprising at least one PEG molecule attached to an attachment
25 group of at least one of the subunits.
6. The conjugate of claim 5, wherein said at least one PEG molecule is bound to the N-terminal of at least one of the subunits.
- 30 7. The conjugate of any of claims 1-6, wherein the amino acid sequence of the FSH- α subunit differs from that of wildtype human FSH- α .

8. The conjugate of any of claims 1-6, wherein the amino acid sequence of the FSH- β subunit differs from that of wildtype human FSH- β .

9. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising

5 i) a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein the amino acid sequence of at least one of said FSH- α and FSH- β subunits differs from that of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced, and

10 ii) at least one oligosaccharide moiety bound to an N-glycosylation site of at least one of said subunits.

10. The conjugate of claim 9, wherein at least one N-glycosylation site has been introduced into the FSH- α subunit by a mutation selected from the group consisting of

P2(a)N+V4(a)S, P2(a)N+V4(a)T, D3(a)N+Q5(a)S, D3(a)N+Q5(a)T, V4(a)N+D6(a)S,

15 V4(a)N+D6(a)S, D6(a)N+P8(a)S, D6(a)N+P8(a)T, E9(a)N+T11(a)S, E9(a)N,

T11(a)N+Q13(a)S, T11(a)N+Q13(a)T, L12(a)N+E14(a)S, L12(a)N+E14(a)T,

E14(a)N+P16(a)S, E14(a)N+P16(a)T, P16(a)N+F18(a)S, P16(a)N+F18(a)T, F17(a)N,

F17(a)N+S19(a)T, G22(a)N+P24(a)S, G22(a)N+P24(a)T, P24(a)N+L26(a)S,

P24(a)N+L26(a)T, F33(a)N+R35(a)S, F33(a)N+R35(a)T, R42(a)N+K44(a)S,

20 R42(a)N+K44(a)T, S43(a)N+K45(a)S, S43(a)N+K45(a)T, K44(a)N+T46(a)S, K44(a)N,

K45(a)N+M47(a)S, K45(a)N+M47(a)T, T46(a)N+L48(a)S, T46(a)N+L48(a)T,

L48(a)N+Q50(a)S, L48(a)N+Q50(a)T, V49(a)N+K51(a)S, V49(a)N+K51(a)T,

Q50(a)N+N52(a)S, Q50(a)N+N52(a)T, V61(a)N+K63(a)S, V61(a)N+K63(a)T,

K63(a)N+Y65(a)S, K63(a)N+Y65(a)T, S64(a)N+N66(a)S, S64(a)N+N66(a)T,

25 Y65(a)N+R67(a)S, Y65(a)N+R67(a)T, V68(a)S, V68(a)T, R67(a)N+T69(a)S, R67(a)N,

T69(a)N+M71(a)S, T69(a)N+M71(a)T, M71(a)N+G73(a)S, M71(a)N+G73(a)T,

G72(a)N+F74(a)S, G72(a)N+F74(a)T, G73(a)N+K75(a)S, G73(a)N+K75(a)T,

F74(a)N+V76(a)S, F74(a)N+V76(a)T, K75(a)N+E77(a)S, K75(a)N+E77(a)T,

A81(a)N+H83(a)S, A81(a)N+H83(a)T, H83(a)N, T86(a)N+Y88(a)S, T86(a)N+Y88(a)T,

30 Y88(a)N+H90(a)S, Y88(a)N+H90(a)T, Y89(a)N+K91(a)S, Y89(a)N+K91(a)T, H90(a)N and

H90(a)N+S92(a)T.

11. The conjugate of claim 9 or 10, wherein at least one N-glycosylation site has been introduced into the FSH- β subunit by a mutation selected from the group consisting of S2(b)N+E4(b)S, S2(b)N+E4(b)T, E4(b)N+T6(b)S, E4(b)N, L5(b)N+N7(b)S, L5(b)N+L7(b)T, T6(b)N+I8(b)S, T6(b)N+I8(b)T, I8(b)N+I10(b)S, I8(b)N+I10(b)T, T9(b)N+A11(b)S, T9(b)N+A11(b)T, K14(b)N+E16(b)S, K14(b)N+E16(b)T, F19(b)N+I21(b)S, F19(b)N+I21(b)T, I21(b)N+I23(b)S, I21(b)N+I23(b)T, S22(b)N+N24(b)S, S22(b)N+N24(b)T, Y31(b)N+Y33(b)S, Y31(b)N+Y33(b)T, Y33(b)N+R35(b)S, Y33(b)N+R35(b)T, R35(b)N+L37(b)S, R35(b)N+L37(b)T, D36(b)N+V38(b)S, D36(b)N+V38(b)T, L37(b)N+Y39(b)S, L37(b)N+Y39(b)T, K40(b)N+P42(b)S, K40(b)N+P42(b)T, A43(b)N+P45(b)S, A43(b)N+P45(b)T, P45(b)N+I47(b)S, P45(b)N+I47(b)T, K46(b)N+Q48(b)S, K46(b)N+Q48(b)T, I47(b)N+K49(b)S, I47(b)N+K49(b)T, K54(b)N+L56(b)S, K54(b)N+L56(b)T, E55(b)N+V57(b)S, E55(b)N+V57(b)T, L56(b)N+Y58(b)S, L56(b)N+Y58(b)T, V57(b)N+E59(b)S, V57(b)N+E59(b)T, Y58(b)N+T60(b)S, Y58(b)N, E59(b)N+V61(b)S, E59(b)N+V61(b)T, T60(b)N+R62(b)S, T60(b)N+R62(b)T, R62(b)N+P64(b)S, R62(b)N+P64(b)T, G65(b)N+A67(b)S, G65(b)N+A67(b)T, A67(b)N+H69(b)S, A67(b)N+H69(b)T, H68(b)N+A70(b)S, H68(b)N+A70(b)T, H69(b)N+D71(b)S, H69(b)N+D71(b)T, D71(b)N+L73(b)S, D71(b)N+L73(b)T, L73(b)N+T75(b)S, L73(b)N, T75(b)N+P77(b)S, T75(b)N+P77(b)T, H83(b)N+G85(b)S, H83(b)N+G85(b)T, K86(b)N+D88(b)S, K86(b)N+D88(b)T, D88(b)N+D90(b)S, D88(b)N+D90(b)T, S89(b)N, S89(b)N+S91(b)T, D90(b)N+T92(b)S, D90(b)N, S91(b)N+D93(b)S, S91(b)N+D93(b)T, D93(b)N+T96(b)S, D93(b)N, T95(b)N+R97(b)S, T95(b)N+R97(b)T, V96(b)N+G98(b)S, V96(b)N+G98(b)T, R97(b)N+L99(b)S, R97(b)N+L99(b)T, L99(b)N+P101(b)S, L99(b)N+P101(b)T, Y103(b)N, Y103(b)N+S105(b)T, S105(b)N+G107(b)S, S105(b)N+G107(b)T, F106(b)N+E108(b)S, F106(b)N+E108(b)T, G107(b)N+M109(b)S, G107(b)N+M109(b)T, E108(b)N+K110(b)S, E108(b)N+K110(b)T, M109(b)N+E111(b)S, and M109(b)N+E111(b)T.

12. The conjugate of any of claims 9-11, wherein at least one of the FSH- α and FSH- β subunits comprises at least one N- or C-terminal peptide addition comprising at least one N-glycosylation site.

13. The conjugate of any of claims 9-12, which further comprises at least one non-polypeptide moiety different from an N- or O-linked oligosaccharide moiety bound to an attachment group of the polypeptide.

5 14. The conjugate of any of claims 9-13, wherein the amino acid sequence of at least one of said FSH- α and FSH- β subunits further differs from that of the corresponding wildtype subunit in that at least one naturally occurring N-glycosylation site has been removed.

10 15. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising an FSH- α subunit and an FSH- β subunit, wherein at least one of said FSH- α and FSH- β subunits comprises a polymer molecule bound to the N-terminal thereof.

15 16. The conjugate of claim 15, wherein the polymer molecule is polyethylene glycol.

17. The conjugate of claim 15 or 16, wherein at least one of said FSH- α and FSH- β subunit comprises, relative to the corresponding wildtype human subunit, at least one introduced amino acid residue comprising an attachment group for the polymer molecule, and/or
20 wherein at least one amino acid residue comprising an attachment group for a polymer molecule has been removed.

18. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a
25 dimeric polypeptide comprising FSH- α and FSH- β subunits, wherein at least one of said FSH- α and FSH- β subunits comprises, relative to the corresponding wildtype subunit, at least one introduced N- or O-glycosylation site at the N-terminal thereof, said at least one introduced glycosylation site being glycosylated.

30 19. The conjugate of claim 18, wherein said at least one introduced N- or O-glycosylation site is part of an N-terminal peptide addition.

20. The conjugate of any of the preceding claims, wherein the FSH- α subunit comprises hFSH- α having the sequence shown in SEQ ID NO:2, or the FSH- β subunit comprises hFSH- β having the sequence shown in SEQ ID NO:4.

5 21. The conjugate of any of the preceding claims, wherein the amino acid sequence of the FSH- α and/or FSH- β subunit differs in 1-20 amino acid residues from that of the corresponding wildtype sequence.

22. The conjugate of any of the preceding claims, which has an increased functional
10 *in vivo* half-life and/or serum half-life as compared to hFSH.

23. The conjugate of any of the preceding claims, wherein the FSH- α subunit and the FSH- β subunit are linked by a peptide bond or a peptide linker to form a single-chain polypeptide; or a single-chain polypeptide conjugate comprising at least two FSH- α subunits
15 or at least two FSH- β subunits, wherein at least one of said subunits differs from the corresponding wildtype FSH subunit as defined in any of the preceding claims.

24. A composition comprising a conjugate according to any of claims 1-23 and at least one pharmaceutically acceptable carrier or excipient.

20 25. Use of conjugate according to any of claims 1-23 or a composition according to claim 24 as a pharmaceutical.

26. Use of a conjugate according to any of claims 1-23 or a composition according
25 to claim 24 for the manufacture of a medicament for treatment of infertility.

27. A method of treating an infertile mammal, comprising administering to a mammal in need thereof an effective amount of a conjugate according to any of claims 1-23 or a composition according to claim 24.

30 28. A modified FSH- α polypeptide subunit having an amino acid sequence that differs from that of the wildtype hFSH- α subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced.

29. A modified FSH- β polypeptide subunit having has an amino acid sequence that differs from that of the wildtype hFSH- β subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced.

5

30. A nucleotide sequence encoding a modified FSH- α polypeptide according to claim 28 and/or a modified FSH- β polypeptide according to claim 29.

31. An expression vector comprising a nucleotide sequence according to claim 30.

10

32. The expression vector of claim 31, comprising a nucleotide sequence encoding (a) a modified FSH- α subunit and a wildtype hFSH- β subunit, (b) a wildtype hFSH- α subunit and a modified FSH- β subunit, or (c) a modified FSH- α subunit and a modified FSH- β subunit.

15

33. A pair of expression vectors, each vector being capable of transfecting a eukaryotic cell, the vectors comprising nucleotide sequences encoding, respectively, a modified FSH- α subunit according to claim 28 and a wildtype FSH- β subunit, a modified FSH- β subunit according to claim 29 and a wildtype FSH- α subunit, or a modified FSH- α subunit according to claim 28 and a modified FSH- β subunit according to claim 29.

20

34. A host cell comprising a nucleotide sequence according to claim 30, an expression vector according to claim 31 or 32, or a pair of expression vectors according to claim 33.

25

35. The host cell of claim 34, which is a eukaryotic cell.

36. The host cell of claim 35, which is a mammalian cell.

37. A method for producing a recombinant heterodimeric FSH protein, comprising subjecting a host cell according to any of claims 34-36 comprising a nucleotide sequence encoding an FSH- α subunit and an FSH- β subunit to cultivation under conditions conducive for expression of said subunits.

30

38. The method of claim 37, wherein the host cell is a eukaryotic cell capable of *in vivo* glycosylation, and the amino acid sequence of at least one of said FSH- α and FSH- β subunits differs from the sequence of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced.

5

39. The method of claim 38, further comprising subjecting the heterodimeric protein to *in vitro* conjugation to a non-polypeptide moiety.

40. The method of claim 39, wherein the non-polypeptide moiety is a polymer moiety such as PEG.

10

1/1

FIGURE 1

Sequence alignment of human FSH to the structural part of two published structures of Human Chorionic Gonadotropin ("1HRP" and "1HCN"). The "/" indicates the chain break between the alpha and the beta chain.

FSH	-QDCPECTLQ	ENPFFSQPGA	PILQCMGCCF	SRAYPTPLRS	KKTMLVQKNV
1HRP	TQDCPECTLQ	ENPFFSQPGA	PILQCMGCCF	SRAYPTPLRS	KKTMLVQKNV
1HCN	-QDCPECTLQ	ENPFFSQPGA	PILQCMGCCF	SRAYPTPLRS	KKTMLVQKNV
FSH	TSESTCCVAK	SYNRVTVMGG	FKVENHTACH	CSTCYY/---	--NSCEL TNI
1HRP	TSESTCCVAK	SYNRVTVMGG	FKVENHTACH	CSTCYY/KEP	LRPRCRPINA
1HCN	TSESTCCVAK	SYNRVTVMGG	FKVENHTACH	CSTCYY/KEP	LRPRCRPINA
FSH	TIAIEKEEER	FCISINTTWC	AGYCYTRDLV	YKDPARPKIQ	KTCTFKELVY
1HRP	TLAVEKEGCP	VCITVNTTIC	AGYCPTMTRV	LQGVLPALPQ	VVCNYRDVRF
1HCN	TLAVEKEGCP	VCITVNTTIC	AGYCPTMTRV	LQGVLPALPQ	VVCNYRDVRF
FSH	ETVRVPGCAH	HADSLYTYPV	ATQCHCGKCD	SDSTDCTVRG	LGPSYCSFGE
1HRP	ESIRLPGCPR	GVNPVVS YAV	ALSCQCALCR	RSTTDCGGPK	DHPLTCD...
1HCN	ESIRLPGCPR	GVNPVVS YAV	ALSCQCALCR	RSTTDCGGPK	DHPLTCD...
FSH	MKE				
1HRP	...				
1HCN	...				

SEQUENCE LISTING

<110> Maxygen ApS

<120> Follicle stimulating hormones

<130> 214wo100

<160> 7

<170> PatentIn version 3.0

<210> 1

<211> 116

<212> PRT

<213> Homo sapiens

<400> 1

```

Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Phe Leu Val Thr Leu Ser
1      5      10
Val Phe Leu His Val Leu His Ser Ala Pro Asp Val Gln Asp Cys Pro
20     25     30
Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro
35     40     45
Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro
50     55     60
Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu
65     70     75
Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly
85     90     95
Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr
100    105    110
Tyr His Lys Ser
115

```

<210> 2

<211> 92

<212> PRT

<213> Homo sapiens

<400> 2

Ala Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro
 1 5 10 15
 Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys
 20 25 30
 Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu
 35 40 45
 Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser
 50 55 60
 Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr
 65 70 75 80
 Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
 85 90

<210> 3

<211> 129

<212> PRT

<213> Homo sapiens

<400> 3

Met Lys Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile
 1 5 10 15
 Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys
 20 25 30
 Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly
 35 40 45
 Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys
 50 55 60
 Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg
 65 70 75 80
 Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val
 85 90 95
 Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys
 100 105 110
 Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys
 115 120 125
 Glu

<210> 4

<211> 111

<212> PRT

<213> Homo sapiens

<400> 4

Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys Glu Glu
 1 5 10 15
 Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly Tyr Cys
 20 25 30
 Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys Ile Gln
 35 40 45
 Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg Val Pro
 50 55 60
 Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val Ala Thr
 65 70 75 80
 Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys Thr Val
 85 90 95
 Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys Glu
 100 105 110

<210> 5

<211> 6186

<212> DNA

<213> Artificial sequence

<220>

<221> exon

<222> (1225)..(1572)

<223> Coding sequence for human FSH-alpha

<400> 5

gacggatcgg gagatctccc gatccccctat ggtcgactct cagtacaatc tgctctgatg 60
 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggagggtcgct gagtagtgcg 120
 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
 ttagggtttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600

tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg	660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc	720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg	780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca	840
ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gctggctagc	900
ttattgcggt agtttatcac agttaaatg ctaacgcagt cagtgccttct gacacaacag	960
tctcgaactt aagctgcagt gactctctta aggtagcctt gcagaagttg gtcgtgaggc	1020
actgggcagg taagtatcaa ggttacaaga caggtttaag gagaccaata gaaactgggc	1080
ttgtcgagac agagaagact cttgcgtttc tgataggcac ctattggctct tactgacatc	1140
cactttgcct ttctctccac aggtgtccac tcccagttca attacagctc ttaaaagctt	1200
ggtaccgagc tcggatccgc cacc atg gac tac tac cgc aag tac gcc gcc	1251
Met Asp Tyr Tyr Arg Lys Tyr Ala Ala	
1 5	
atc ttc ctg gtg acc ctg agc gtg ttc ctg cac gtg ctg cac agc gcc	1299
Ile Phe Leu Val Thr Leu Ser Val Phe Leu His Val Leu His Ser Ala	
10 15 20 25	
ccc gac gtg cag gac tgc ccc gag tgc acc ctg cag gag aac ccc ttc	1347
Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe	
30 35 40	
ttc agc cag ccc ggc gcc ccc atc ctg cag tgc atg ggc tgc tgc ttc	1395
Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe	
45 50 55	
agc cgc gcc tac ccc acc ccc ctg cgc agc aag aag acc atg ctg gtg	1443
Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val	
60 65 70	
cag aag aac gtg acc agc gag agc acc tgc tgc gtg gcc aag agc tac	1491
Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr	
75 80 85	
aac cgc gtg acc gtg atg ggc ggc ttc aag gtg gag aac cac acc gcc	1539
Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala	
90 95 100 105	
tgc cac tgc agc acc tgc tac tac cac aag agc taatctagag ggcccgttta	1592
Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser	
110 115	
aacccgctga tcagcctcga ctgtgccttc tagttgccag ccatctgttg tttgcccctc	1652
ccccgtgcct tccttgaccc tggaagggtgc cactcccact gtcctttcct aataaaatga	1712
ggaaattgca tcgcattgtc tgagtaggtg tcattctatt ctgggggggtg ggggtgggca	1772
ggacagcaag ggggaggatt gggaagacaa tagcaggcat gctggggatg cgggtgggctc	1832
tatggcttct gaggcggaaa gaaccagctg gggctctagg gggatatccc acgcgccctg	1892
tagcggcgca ttaagcgcg cggtgtggt ggttacgcgc agcgtgaccg ctacacttgc	1952
cagcgcccta gcgccgctc ctttcgcttt cttcccttcc tttctcgcca cgttcgccgg	2012
ctttccccgt caagctctaa atcggggcat ccctttaggg ttccgattta gtgctttacg	2072

gcacctcgac cccaaaaaac ttgattaggg tgatgggttca cgtagtgggc catcgccctg 2132
atagacgggt tttcgccctt tgacgttggg gtccacgttc tttaatagtg gactcttggt 2192
ccaaactgga acaacactca accctatctc ggtctattct tttgatttat aagggatttt 2252
ggggatttcg gcctattggg taaaaaatga gctgatttaa caaaaattta acgcgaatta 2312
attctgtgga atgtgtgtca gttaggggtg ggaaagtccc caggctcccc aggcaggcag 2372
aagtatgcaa agcatgcatc tcaattagtc agcaaccagg tgtggaaagt ccccaggctc 2432
cccagcaggc agaagtatgc aaagcatgca tctcaattag tcagcaacca tagtcccgcc 2492
cctaactccg cccatcccgc ccctaactcc gccagttcc gccattctc cgccccatgg 2552
ctgactaatt ttttttattt atgcagaggc cgaggccgcc tctgcctctg agctattcca 2612
gaagtagtga ggaggctttt ttggaggcct aggcttttgc aaaaagctcc cgggagcttg 2672
tatatccatt ttcggatctg atcagcacgt gatgaaaaag cctgaactca ccgcgacgtc 2732
tgtcgagaag tttctgatcg aaaagttcga cagcgtctcc gacctgatgc agctctcgga 2792
gggcaagaa tctcgtgctt tcagcttcga tgtaggaggg cgtggatatg tcctgcgggt 2852
aaatagctgc gccgatgggt tctacaaaga tcgttatgtt tatcggcact ttgcatcggc 2912
cgcgctcccg attccggaag tgcttgacat tggggaattc agcgagagcc tgacctattg 2972
catctcccgc cgtgcacagg gtgtcacgtt gcaagacctg cctgaaaccg aactgcccgc 3032
tgttctgcag ccggctgcgg aggccatgga tgcgatcgct gcggccgatc ttagccagac 3092
gagcgggttc ggccatttcg gaccgcaagg aatcgggtcaa tacactacat ggcgtgattt 3152
catatgcgcg attgctgatc cccatgtgta tcaactggcaa actgtgatgg acgacaccgt 3212
cagtgcgtcc gtcgcgcagg ctctcgatga gctgatgctt tgggcccagg actgccccga 3272
agtccggcac ctcgtgcacg cggatttcgg ctccaacaat gtcctgacgg acaatggccg 3332
cataacagcg gtcattgact ggagcgaggc gatgttcggg gattcccaat acgaggtcgc 3392
caacatcttc ttctggaggc cgtggttggc ttgtatggag cagcagacgc gctacttcga 3452
gcgaggcat ccggagcttg caggatcgcc gcggctccgg gcgtatatgc tccgatttg 3512
tcttgaccaa ctctatcaga gcttgggtga cggcaatttc gatgatgcag cttgggcgca 3572
gggtcgatgc gacgcaatcg tccgatccgg agccgggact gtcgggcgta cacaatcgc 3632
ccgcagaagc gcggccgtct ggaccgatgg ctgtgtagaa gtactcgccg atagtggaaa 3692
ccgacgcccc agcactcgtc cgagggcaaa ggaatagcac gtgctacgag atttcgattc 3752
caccgcccgc ttctatgaaa gggtgggctt cggaatcggt ttccgggacg ccggctggat 3812
gatcctccag cgcggggatc tcatgctgga gttcttcgcc caccccaact tgtttattgc 3872
agcttataat gggtacaaat aaagcaatag catcacaat ttcacaaata aagcattttt 3932
ttcactgcat tctagttgtg gtttgtccaa actcatcaat gtatcttatc atgtctgtat 3992
accgtcgacc tctagctaga gcttggcgta atcatgggtca tagctgtttc ctgtgtgaaa 4052
ttgttatccg ctcaaatc cacacaacat acgagccgga agcataaagt gtaaagcctg 4112

gggtgcctaa	tgagtgaact	aactcacatt	aattgcgttg	cgctcactgc	ccgctttcca	4172
gtcgggaaac	ctgtcgtgcc	agctgcatta	atgaatcggc	caacgcgcgg	ggagaggcgg	4232
tttgcgtatt	gggcgcctct	ccgcttcctc	gctcactgac	tcgctgcgct	cggtcgttcg	4292
gctgcggcga	gcggtatcag	ctcactcaaa	ggcggtaata	cggttatcca	cagaatcagg	4352
ggataacgca	ggaaagaaca	tgtgagcaaa	aggccagcaa	aaggccagga	accgtaaaaa	4412
ggccgcgttg	ctggcgcttt	tccataggct	ccgccccct	gacgagcatc	acaaaaatcg	4472
acgctcaagt	cagagggtgg	gaaacccgac	aggactataa	agataccagg	cgtttcccc	4532
tggaagctcc	ctcgtgcgct	ctcctgttcc	gaccctgccg	cttaccggat	acctgtccgc	4592
ctttctccct	tcgggaagcg	tggcgctttc	tcaatgctca	cgctgtaggt	atctcagttc	4652
ggtgtaggtc	gttcgctcca	agctgggctg	tgtgcacgaa	cccccgcttc	agcccgaccg	4712
ctgcgcctta	tccggtaact	atcgtcttga	gtccaacccg	gtaagacacg	acttatcgcc	4772
actggcagca	gccactggta	acaggattag	cagagcgagg	tatgtaggcg	gtgctacaga	4832
gttcttgaag	tggtggccta	actacggcta	cactagaagg	acagtatttg	gtatctgcgc	4892
tctgctgaag	ccagttacct	tcggaaaaag	agttggtagc	tcttgatccg	gcaaacaac	4952
caccgctggg	agcggtggtt	tttttggttg	caagcagcag	attacgcgca	gaaaaaagg	5012
atctcaagaa	gaccccttga	tcttttctac	ggggtctgac	gctcagtggg	acgaaaactc	5072
acgttaaggg	attttggtca	tgagattatc	aaaaaggatc	ttcacctaga	tccttttaaa	5132
ttaaaaatga	agtttttaaa	caatctaaag	tatatatgag	taaacttggg	ctgacagtta	5192
ccaatgctta	atcagtgagg	cacctatctc	agcgatctgt	ctatttcggt	catccatagt	5252
tgcctgactc	cccgtcgtgt	agataactac	gatacgggag	ggcttaccat	ctggccccag	5312
tgctgcaatg	ataccgcgag	accacgctc	accggctcca	gatttatcag	caataaacca	5372
gccagccgga	agggccgagc	gcagaagtgg	tcctgcaact	ttatccgcct	ccatccagtc	5432
tattaattgt	tgccgggaag	ctagagtaag	tagttcgcca	gttaatagtt	tgcgcaacgt	5492
tgttgccatt	gctacaggca	tcgtgggtgt	acgctcgtcg	tttggtatgg	cttcattcag	5552
ctccggttcc	caacgatcaa	ggcgagttac	atgatcccc	atgttggtgca	aaaaagcggg	5612
tagctccttc	ggtcctccga	tcgttgctcag	aagtaagttg	gccgcagtgt	tatcactcat	5672
ggttatggca	gcactgcata	attctcttac	tgtcatgcca	tccgtaagat	gcttttctgt	5732
gactggtgag	tactcaacca	agtcattctg	agaatagtgt	atgcggcgac	cgagttgctc	5792
ttgcccggcg	tcaatacggg	ataataccgc	gccacatagc	agaactttta	aagtgtcat	5852
cattggaaaa	cgttcttcgg	ggcgaaaact	ctcaaggatc	ttaccgctgt	tgagatccag	5912
ttcgatgtaa	ccactcgtg	caccctaactg	atcttcagca	tcttttactt	tcaccagcgt	5972
ttctgggtga	gcaaaaacag	gaaggcaaaa	tgccgcaaaa	aagggaataa	gggcgacacg	6032
gaaatgttga	atactcatal	tcttcctttt	tcaatattat	tgaagcattt	atcagggtta	6092
ttgtctcatg	agcggataca	tatttgaatg	tatttagaaa	aataaaca	taggggttcc	6152

gcgcacattt ccccgaaaag tgccacctga cgtc 6186

<210> 6

<211> 5651

<212> DNA

<213> Artificial sequence

<220>

<221> exon

<222> (1231)..(1617)

<223> Coding sequence for human FSH-beta

<400> 6

gacggatcgg gagatctccc gatccccctat ggtcgactct cagtacaatc tgctctgatg	60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg	120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc	180
ttagggttag gcgtttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt	240
gattattgac tagttattaa tagtaatcaa ttacgggggc attagttcat agcccatata	300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc	360
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc	420
attgacgtca atgggtggac tatttacggt aaactgccc cttggcagta catcaagtgt	480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt	540
atgccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca	600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg	660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc	720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc ccatttgacg caaatgggcg	780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca	840
ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gctggctagc	900
ttattgcggt agtttatcac agttaaatg ctaacgcagt cagtgtttct gacacaacag	960
tctcgaactt aagctgcagt gactctctta aggtagcctt gcagaagttg gtcgtgaggc	1020
actgggcagg taagtatcaa gggtacaaga caggtttaag gagaccaata gaaactgggc	1080
ttgtcgagac agagaagact cttgcgtttc tgataggcac ctattgggtct tactgacatc	1140
cactttgcct ttctctccac aggtgtccac tcccagttca attacagctc ttaaaagctt	1200
ggtagcgagc tcggatctat cgatgccacc atg gag acc ctg cag ttc ttc ttc	1254

																Met 1	Glu 2	Thr 3	Leu 4	Gln 5	Phe 6	Phe 7	Phe 8	
ctg Leu	ttc Phe 10	tgc Cys	tgc Cys	tgg Trp	aag Lys	gcc Ala 15	atc Ile	tgc Cys	tgc Cys	aac Asn	agc Ser 20	tgc Cys	gag Glu	ctg Leu	acc Thr	1302								
aac Asn 25	atc Ile	acc Thr	atc Ile	gcc Ala	atc Ile 30	gag Glu	aag Lys	gag Glu	gag Glu	tgc Cys 35	cgc Arg	ttc Phe	tgc Cys	atc Ile	agc Ser 40	1350								
atc Ile	aac Asn	acc Thr	acc Thr	tgg Trp 45	tgc Cys	gcc Ala	ggc Gly	tac Tyr	tgc Cys 50	tac Tyr	acc Thr	cgc Arg	gac Asp	ctg Leu 55	gtg Val	1398								
tac Tyr	aag Lys	gac Asp	ccc Pro 60	gcc Ala	cgc Arg	ccc Pro	aag Lys	atc Ile 65	cag Gln	aag Lys	acc Thr	tgc Cys 70	acc Thr	ttc Phe	aag Lys	1446								
gag Glu	ctg Leu	gtg Val 75	tac Tyr	gag Glu	acg Thr	gtc Val	cgg Arg 80	gtg Val	ccc Pro	ggc Gly	tgc Cys	gcc Ala 85	cac His	cac His	gcc Ala	1494								
gac Asp	agc Ser 90	ctg Leu	tac Tyr	acc Thr	tac Tyr	ccc Pro 95	gtg Val	gcc Ala	acc Thr	cag Gln	tgc Cys 100	cac His	tgc Cys	ggc Gly	aag Lys	1542								
tgc Cys 105	gac Asp	agc Ser	gac Asp	agc Ser	acc Thr 110	gac Asp	tgc Cys	acc Thr	gtg Val	cgc Arg 115	ggc Gly	ctg Leu	ggc Gly	ccc Pro	agc Ser 120	1590								
tac Tyr	tgc Cys	agc Ser	ttc Phe	ggc Gly 125	gag Glu	atg Met	aag Lys	gag Glu	taactcgaga ctagagggcc							1637								
cgtttaaacc			cgctgatcag			cctcgactgt			gccttctagt			tgccagccat			ctgttgtttg		1697							
cccctcccc			gtgccttcct			tgaccctgga			aggtgccact			ccactgtcc			tttcctaata		1757							
aaatgaggaa			attgcatcgc			attgtctgag			taggtgtcat			tctattctgg			ggggtggggt		1817							
ggggcaggac			agcaaggggg			aggattggga			agacaatagc			aggcatgctg			gggatgcggt		1877							
gggctctatg			gcttctgagg			cggaaagaac			cagctggggc			tctaggggggt			atccccacgc		1937							
gccctgtagc			ggcgcattaa			gcgcggcggg			tgtggtgggt			acgcgcagcg			tgaccgctac		1997							
acttgccagc			gccctagcgc			ccgctccttt			cgctttcttc			ccttcctttc			tcgccacgtt		2057							
cgccggcttt			ccccgtcaag			ctctaaatcg			gggcatccct			ttagggttcc			gatttagtgc		2117							
tttacggcac			ctcgacccca			aaaaacttga			ttaggggtgat			ggttcacgta			gtggggccatc		2177							
gccctgatag			acggtttttc			gccctttgac			gttggagtcc			acgttcttta			atagtggact		2237							
cttgttccaa			actggaacaa			cactcaaccc			tatctcggtc			tattcttttg			atttataagg		2297							
gattttgggg			atttcggcct																					

attccagaag	tagtgaggag	gcttttttgg	aggcctaggc	ttttgcaaaa	agctcccggg	2717
agcttgtata	tccattttcg	gatctgatca	gcacgtgttg	acaattaatc	atcggcatag	2777
tatatcggca	tagtataata	cgacaagggtg	aggaactaaa	ccatggccaa	gttgaccagt	2837
gccgttccgg	tgctcaccgc	gcgcgacgtc	gccggagcgg	tcgagttctg	gaccgaccgg	2897
ctcgggttct	cccgggactt	cgtggaggac	gacttcgccg	gtgtgggtccg	ggacgacgtg	2957
accctgttca	tcagcgcggt	ccaggaccag	gtggtgccgg	acaacaccct	ggcctgggtg	3017
tgggtgcgcg	gcctggacga	gctgtacgcc	gagtggtcgg	aggtcgtgtc	cacgaacttc	3077
cgggacgcct	ccggggccggc	catgaccgag	atcggcgagc	agccgtgggg	gcgggagttc	3137
gccctgcgcg	acccggccgg	caactgcgtg	cacttcgtgg	ccgaggagca	ggactgacac	3197
gtgctacgag	atttcgattc	caccgccgcc	ttctatgaaa	ggttgggctt	cggaatcgtt	3257
ttccgggacg	ccggctggat	gatcctccag	cgcggggatc	tcatgctgga	gttcttcgcc	3317
caccccaact	tgttttattgc	agcttataat	ggttacaaat	aaagcaatag	catcacaaat	3377
ttcacaaata	aagcattttt	ttcactgcat	tctagttgtg	gtttgtccaa	actcatcaat	3437
gtatcttatc	atgtctgtat	accgtcgacc	tctagctaga	gcttggcgta	atcatggtca	3497
tagctgtttc	ctgtgtgaaa	ttgttatccg	ctcacaaatc	cacacaacat	acgagccgga	3557
agcataaagt	gtaaagcctg	gggtgcctaa	tgagtgaagt	aactcacatt	aattgcgttg	3617
cgctcactgc	ccgctttcca	gtcgggaaac	ctgtcgtgcc	agctgcatta	atgaatcggc	3677
caacgcgcgg	ggagaggcgg	tttgcgattt	gggcgtcttt	ccgcttcctc	gctcactgac	3737
tcgctgcgct	cggctgttcg	gctgcggcga	gcggtatcag	ctcactcaaa	ggcggtaata	3797
cggttatcca	cagaatcagg	ggataacgca	ggaaagaaca	tgtgagcaaa	aggccagcaa	3857
aaggccagga	accgtaaaaa	ggccgcgttg	ctggcgtttt	tccataggct	ccgccccctt	3917
gacgagcatc	acaaaaatcg	acgctcaagt	cagaggtggc	gaaacccgac	aggactataa	3977
agataccagg	cgtttcccc	tggaagctcc	ctcgtgcgct	ctcctgttcc	gaccctgccg	4037
cttaccggat	acctgtccgc	ctttctccct	tcgggaagcg	tggcgctttc	tcaatgctca	4097
cgctgtaggt	atctcagttc	gggtgtaggtc	gttcgctcca	agctgggctg	tgtgcacgaa	4157
cccccgttc	agcccgaccg	ctgcgcctta	tccggtaact	atcgtcttga	gtccaacccg	4217
gtaagacacg	acttatcgcc	actggcagca	gccactggta	acaggattag	cagagcgagg	4277
tatgtaggcg	gtgctacaga	gttcttgaag	tgggtggccta	actacggcta	cactagaagg	4337
acagtatttg	gtatctgcgc	tctgctgaag	ccagttacct	tcggaaaaag	agttggtagc	4397
tcttgatccg	gcaaacaaac	caccgctggg	agcgggtggtt	tttttgtttg	caagcagcag	4457
attacgcgca	gaaaaaaaag	atctcaagaa	gatcctttga	tcttttctac	ggggtctgac	4517
gctcagtggg	acgaaaactc	acgttaaggg	attttggtca	tgagattatc	aaaaaggatc	4577
ttcacctaga	tcctttttaa	ttaaaaatga	agtttttaaat	caatctaaag	tatatatgag	4637
taaacttggt	ctgacagtta	ccaatgctta	atcagtgagg	cacctatctc	agcgatctgt	4697

```

ctatttcggt catccatagt tgcctgactc cccgtcgtgt agataactac gatacgggag 4757
ggcttaccat ctggccccag tgctgcaatg ataccgagag acccacgctc accgggtcca 4817
gatttatcag caataaacca gccagccgga agggccgagc gcagaagtgg tcctgcaact 4877
ttatccgcct ccatccagtc tattaattgt tgccgggaag ctagagtaag tagttcgcca 4937
gttaatagtt tgcgcaacgt tggtgccatt gctacaggca tcgtggtgtc acgctcgtcg 4997
tttggtatgg cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatcccc 5057
atgttggtgca aaaaagcggg tagctccttc ggtcctccga tcgttggtcag aagtaagttg 5117
gccgcagtgt tatcactcat ggttatggca gcactgcata attctcttac tgtcatgcca 5177
tccgtaagat gcttttctgt gactggtgag tactcaacca agtcattctg agaatagtgt 5237
atgcgggcgac cgagttgctc ttgcccggcg tcaatacggg ataataccgc gccacatagc 5297
agaactttta aagtgtctcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc 5357
ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg caccacaactg atcttcagca 5417
tcttttactt tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa 5477
aagggaataa gggcgacacg gaaatgttga atactcatac tcttcctttt tcaatattat 5537
tgaagcattt atcaggggta ttgtctcatg agcggataca tatttgaaatg tatttagaaa 5597
aataaacaaa taggggttcc gcgcacattt ccccgaaaag tgccacctga cgtc 5651

```

<210> 7

<211> 6213

<212> DNA

<213> Artificial sequence

<220>

<221> exon

<222> (1225)..(1599)

<223> Coding sequence for modified FSH- α pha

<400> 7

```

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttaggggttag gcgttttgcg ctgcttcgag atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacgggggc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360

```

cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc	420
attgacgtca atgggtggac tatttacggt aaactgcca cttggcagta catcaagtgt	480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt	540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca	600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg	660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc	720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg	780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca	840
ctgcttactg gcttatcgaa attaatacga ctactatag ggagacccaa gctggctagc	900
ttattgcggt agtttatcac agttaaattg ctaacgcagt cagtgccttct gacacaacag	960
tctcgaactt aagctgcagt gactctctta aggtagcctt gcagaagttg gtcgtgaggc	1020
actgggcagg taagtatcaa ggttacaaga caggtttaag gagaccaata gaaactgggc	1080
ttgtcgagac agagaagact cttgcgtttc tgataggcac ctattggtct tactgacatc	1140
cactttgcct ttctctccac aggtgtccac tcccagttca attacagctc ttaaaagctt	1200
ggtaccgagc tcggatccgc cacc atg gac tac tac cgc aag tac gcc gcc	1251
Met Asp Tyr Tyr Arg Lys Tyr Ala Ala	
1 5	
atc ttc ctg gtg acc ctg agc gtg ttc ctg cac gtg ctg cac agc gcc	1299
Ile Phe Leu Val Thr Leu Ser Val Phe Leu His Val Leu His Ser Ala	
10 15 20 25	
aac atc acc gtt aac atc acc gtg gcc ccc gac gtg cag gac tgc ccc	1347
Asn Ile Thr Val Asn Ile Thr Val Ala Pro Asp Val Gln Asp Cys Pro	
30 35 40	
gag tgc acc ctg cag gag aac ccc ttc ttc agc cag ccc ggc gcc ccc	1395
Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro	
45 50 55	
atc ctg cag tgc atg ggc tgc tgc ttc agc cgc gcc tac ccc acc ccc	1443
Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro	
60 65 70	
ctg cgc agc aag aag acc atg ctg gtg cag aag aac gtg acc agc gag	1491
Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu	
75 80 85	
agc acc tgc tgc gtg gcc aag agc tac aac cgc gtg acc gtg atg ggc	1539
Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly	
90 95 100 105	
ggc ttc aag gtg gag aac cac acc gcc tgc cac tgc agc acc tgc tac	1587
Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr	
110 115 120	
tac cac aag agc taatctagag ggcccgttta aaccgctga tcagcctcga	1639
Tyr His Lys Ser	
125	
ctgtgccttc tagttgccag ccatctgttg tttgccctc ccccgctgct tccttgaccc	1699
tggaagggtgc cactcccact gtcctttcct aataaaatga ggaaattgca tcgcattgtc	1759

tgagtaggtg	tcattctatt	ctggggggtg	gggtggggca	ggacagcaag	ggggaggatt	1819
gggaagacaa	tagcaggcat	gctggggatg	cgggtgggctc	tatggcttct	gaggcgga	1879
gaaccagctg	gggctctagg	gggtatcccc	acgcgccctg	tagcggcgca	ttaagcgcg	1939
cgggtgtggt	ggttacgcgc	agcgtgaccg	ctacacttgc	cagcgcccta	gcgcccgtc	1999
ctttcgcttt	cttcccttcc	tttctcgcca	cgttcgccgg	ctttccccgt	caagctctaa	2059
atcggggcat	ccctttagg	ttccgattta	gtgctttacg	gcacctcgac	cccaaaaaac	2119
ttgattagg	tgatggttca	cgtagtgggc	catcgccctg	atagacgggt	tttcgcccct	2179
tgacgttga	gtccacgttc	tttaatagtg	gactcttggt	ccaaactgga	acaacactca	2239
accctatctc	ggtctattct	tttgatttat	aagggtat	ggggatttcg	gcctattggt	2299
taaaaaatga	gctgatttaa	caaaaattta	acgcgaatta	attctgtgga	atgtgtgtca	2359
gttaggggtg	ggaaagtccc	caggctcccc	aggcaggcag	aagtatgcaa	agcatgcatc	2419
tcaattagtc	agcaaccagg	tgtggaaagt	ccccaggctc	cccagcaggc	agaagtatgc	2479
aaagcatgca	tctcaattag	tcagcaacca	tagtcccgcc	cctaactccg	cccatcccgc	2539
ccctaactcc	gcccagttcc	gcccattctc	cgccccatgg	ctgactaatt	ttttttat	2599
atgcagaggc	cgaggccgcc	tctgcctctg	agctattcca	gaagtagtga	ggaggctttt	2659
ttggaggcct	aggcttttgc	aaaaagctcc	cgggagcttg	tatatccatt	ttcggatctg	2719
atcagcacgt	gatgaaaaag	cctgaactca	ccgcgacgtc	tgtcgagaag	tttctgatcg	2779
aaaagttcga	cagcgtctcc	gacctgatgc	agctctcgga	gggcgaagaa	tctcgtgctt	2839
tcagcttcga	tgtaggaggg	cgtggatatg	tcctgcggg	aaatagctgc	gccgatgggt	2899
tctacaaaga	tcgttatggt	tatcggcact	ttgcatcggc	cgcgctcccc	attccggaag	2959
tgcttgacat	tggggaattc	agcgagagcc	tgacctattg	catctcccgc	cgtgcacagg	3019
gtgtcacgtt	gcaagacctg	cctgaaaccg	aactgcccgc	tgttctgcag	ccggtcgcgg	3079
aggccatgga	tgcatcgct	gcggccgac	ttagccagac	gagcgggttc	ggccattcgc	3139
gaccgcaagg	aatcggtcaa	tacactacat	ggcgtgattt	catatgcgcg	attgctgatc	3199
cccattgtga	tactggcaa	actgtgatgg	acgacaccgt	cagtgcgtcc	gtcgcgcagg	3259
ctctcgatga	gctgatgctt	tgggccgagg	actgccccga	agtccggcac	ctcgtgcacg	3319
cggatttcgg	ctccaacaat	gtcctgacgg	acaatggccg	cataacagcg	gtcattgact	3379
ggagcgaggc	gatgttcggg	gattcccaat	acgaggtcgc	caacatcttc	ttctggaggc	3439
cgtggttggc	ttgtatggag	cagcagacgc	gctacttcga	gcggaggcat	ccggagcttg	3499
caggatcgcc	gcggctccgg	gcgtatatgc	tccgcattgg	tcttgaccaa	ctctatcaga	3559
gcttggttga	cggcaatttc	gatgatgcag	cttgggcgca	gggtcgatgc	gacgcaatcg	3619
tccgatccgg	agccgggact	gtcgggcgta	cacaaatcgc	ccgcagaagc	gcggccgtct	3679
ggaccgatgg	ctgtgtagaa	gtactcgccg	atagtggaaa	ccgacgcccc	agcactcgtc	3739
cgagggcaaa	ggaatagcac	gtgctacgag	atttcgattc	caccgcccgc	ttctatgaaa	3799

ggttgggctt	cggaatcggt	ttccgggacg	ccggctggat	gatcctccag	cgcggggatc	3859
tcatgctgga	gttcttcgcc	cacccaact	tgtttattgc	agcttataat	ggttacaaat	3919
aaagcaatag	catcacaaat	ttcacaaata	aagcattttt	ttcactgcat	tctagtgtgtg	3979
gtttgtccaa	actcatcaat	gtatcttatc	atgtctgtat	accgtcgacc	tctagctaga	4039
gcttggcgta	atcatggtca	tagctgtttc	ctgtgtgaaa	ttgttatccg	ctcacaattc	4099
cacacaacat	acgagccgga	agcataaagt	gtaaagcctg	gggtgcctaa	tgagtgaagt	4159
aactcacatt	aattgcgttg	cgctcactgc	ccgctttcca	gtcgggaaac	ctgtcgtgcc	4219
agctgcatta	atgaatcggc	caacgcgcgg	ggagaggcgg	tttgcgtatt	gggcgctctt	4279
ccgcttcctc	gctcactgac	tcgctgcgct	cggtcgttcg	gctgcggcga	gcggtatcag	4339
ctcactcaaa	ggcggtaata	cggttatcca	cagaatcagg	ggataacgca	ggaaagaaca	4399
tgtgagcaaa	aggccagcaa	aaggccagga	accgtaaaaa	ggccgcgttg	ctggcgtttt	4459
tccataggct	ccgccccctt	gacgagcatc	acaaaaatcg	acgctcaagt	cagagggtggc	4519
gaaacccgac	aggactataa	agataaccagg	cgtttccccc	tggaagctcc	ctcgtgcgct	4579
ctcctgttcc	gaccctgccg	cttaccggat	acctgtccgc	ctttctccct	tcgggaagcg	4639
tggcgctttc	tcaatgctca	cgctgtaggt	atctcagttc	gggtgtaggtc	gttcgctcca	4699
agctgggctg	tgtgcacgaa	ccccccgttc	agcccgaccg	ctgcgcctta	tccggtaact	4759
atcgtcttga	gtccaacccg	gtaagacacg	acttatcgcc	actggcagca	gccactggta	4819
acaggattag	cagagcgagg	tatgtaggcg	gtgctacaga	gttcttgaag	tggtggccta	4879
actacggcta	cactagaagg	acagtatttg	gtatctgcgc	tctgctgaag	ccagttacct	4939
tcggaaaaag	agttggtagc	tcttgatccg	gcaaacaac	caccgctggt	agcggtggtt	4999
tttttgtttg	caagcagcag	attacgcgca	gaaaaaaagg	atctcaagaa	gatcctttga	5059
tcttttctac	ggggctctgac	gctcagtggg	acgaaaactc	acgttaaggg	attttgggtca	5119
tgagattatc	aaaaaggatc	ttcacctaga	tccttttaaa	ttaaaaatga	agttttaaat	5179
caatctaaag	tatatatgag	taaacttggg	ctgacagtta	ccaatgctta	atcagtgagg	5239
cacctatctc	agcgatctgt	ctatttcggt	catccatagt	tgcttgactc	cccgtcgtgt	5299
agataactac	gatacgggag	ggcttaccat	ctggccccag	tgctgcaatg	ataccgcgag	5359
accacgctc	accggctcca	gatttatcag	caataaacca	gccagccgga	agggccgagc	5419
gcagaagtgg	tcctgcaact	ttatccgcct	ccatccagtc	tattaattgt	tgccgggaag	5479
ctagagtaag	tagttcgcca	gttaatagtt	tgcgcaacgt	tggttgccatt	gctacaggca	5539
tcgtggtgtc	acgctcgtcg	tttggtatgg	cttcattcag	ctccggttcc	caacgatcaa	5599
ggcgagttac	atgatcccc	atgttggtga	aaaaagcggg	tagctccttc	ggctctccga	5659
tcgttgctcag	aagtaagttg	gccgcagtgt	tatcactcat	ggttatggca	gactgcata	5719
attctcttac	tgtcatgcca	tccgtaagat	gcttttctgt	gactgggtgag	tactcaacca	5779
agtcattctg	agaatagtgt	atgcggcgac	cgagttgctc	ttgcccggcg	tcaatacggg	5839

ataataccgc gccacatagc agaactttaa aagtgctcat cattggaaaa cgttcttcgg	5899
ggcgaaaact ctcaaggatc ttaccgctgt tgagatccag ttcgatgtaa ccactcgtg	5959
caccaactg atcttcagca tcttttactt tcaccagcgt ttctgggtga gcaaaaacag	6019
gaaggcaaaa tgccgcaaaa aagggaataa gggcgacacg gaaatgttga atactcatac	6079
tcttcctttt tcaatattat tgaagcattt atcagggtta ttgtctcatg agcggataca	6139
tatttgaatg tatttagaaa aataaacaata taggggttcc gcgcacattt ccccgaaaag	6199
tgccaccta cgta	6213

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 01/00090

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 47/48, A61K 38/24, C07K 14/59

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO INTERNAL, WPI DATA, PAJ, BIOSIS, CHEM.ABS.DATA, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9913081 A1 (AKZO NOBEL N.V.), 18 March 1999 (18.03.99), page 2, line 13 - line 17; page 5, line 23 - line 32	1-3,7-12, 18-40
Y	--	4-6,13-17
Y	US 4904584 A (SHAW), 27 February 1990 (27.02.90), column 1, line 16 - line 27; column 2, line 46	4-6,13-17
A	EP 0370205 A2 (AKZO NOBEL N.V.), 30 May 1990 (30.05.90), page 6, line 49 - line 52; page 7, line 4 - line 6	1-40
	-- -----	

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

9 July 2001

Date of mailing of the international search report

25.07.01

Name and mailing address of the International Searching Authority
European Patent Office P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel(+31-70)340-2040, Tx 31 651 epo nl,
Fax(+31-70)340-3016

Authorized officer

Carl-Olof Gustafsson/BS
Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK01/00090

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 27
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet*
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

★

Claim 27 relates to a method of treatment of the human or animal body by surgery or by therapy/a diagnostic method practised on the human or animal body/Rule 39.1(iv). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compounds/compositions.

**

As stated by PCT Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

This application relates to polypeptide conjugates exhibiting FSH activity and having an increased serum half-life as compared to hFSH. The FSH polypeptide has been modified compared to the wildtype polypeptide in that an amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed. Said attachment group is selected from the group consisting of an N- or O-glycosylation site and an attachment group for a polymer molecule, a lipophilic compound, a carbohydrate moiety or an organic derivatising agent.

However document WO 99/13081 shows that the modification of FSH by the introduction or removal of glycosylation sites is generally known in the art and that the glycosylation sites are important for the biological activity, e. g. the circulatory half-life of the hormone.

Therefore, said attachment groups do not represent such a common feature which can be considered a special technical feature within the meaning of PCT Rule 13.2. Therefore, the present application is considered to lack unity of invention, and the following inventions were found:

Invention 1: A polypeptide conjugate exhibiting FSH activity, comprising an introduced N- or O-glycosylation site.

.../...

Invention 2: A polypeptide conjugate exhibiting FSH activity, comprising an attachment group for a polymer molecule, a lipophilic compound, a carbohydrate moiety or an organic derivatising agent.

However, a search has been performed for both inventions 1 and 2.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/07/01

International application No.

PCT/DK 01/00090

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9913081	A1	18/03/99	AU	9538198 A	29/03/99
				BR	9812043 A	03/10/00
				CN	1269834 T	11/10/00
				EP	1012290 A	28/06/00
				HU	0004599 A	28/04/01
				NO	20001178 A	05/05/00
				PL	339259 A	04/12/00
				TR	200000618 T	00/00/00
				ZA	9807497 A	23/02/99
US	4904584	A	27/02/90	AU	2911189 A	19/07/89
				EP	0355142 A	28/02/90
				WO	8905824 A	29/06/89
EP	0370205	A2	30/05/90	AT	168699 T	15/08/98
				DE	68928751 D,T	15/04/99
				ES	2121734 T	16/12/98
				JP	2227075 A	10/09/90
				JP	2928287 B	03/08/99
				US	5218092 A	08/06/93